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Unravelling the molecular and evolutionarymechanisms of copper resistance in plant-associated *Pseudomonas* spp.

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Abstract

Copper (Cu) is a vital trace element for all living organisms but can be extremely harmful when in excess. Its antimicrobial properties have made copper compounds a popular choice in agriculture for managing plant diseases. However, the widespread use of copper-based bactericides has resulted in heavy metal pollution and the emergence of copper-resistant strains, which could potentially undermine disease control efforts. The mechanism by which copper inhibits pathogenic infection, the capacity of pseudomonads to evolve copper resistance, and the potential for co-evolution of resistance to copper and antibiotics are yet to be evaluated. Furthermore, the impact of the evolution of copper resistance on bacterial virulence remains a mystery.

The central objective of my research project was to unravel the molecular and evolutionary underpinnings of copper resistance in pseudomonads, a group of bacteria that are closely associated with plants. This includes the study of *Pseudomonas fluorescens* SBW25, which is a model bacterium known for promoting plant growth. Additionally, my project also focused on *Pseudomonas syringae* pv. *actinidiae* (*Psa*) NZ13 and NZ47, the causative agent of bacterial canker in kiwifruit. By understanding the mechanisms of copper resistance in these bacteria, we can gain insights into how they adapt to environmental stressors, which could have significant implications for agricultural practices and disease management.

In this work, I successfully devised a strategy to quantify bacterial infection using *lux*-labelled bioreporter strains. This innovative technique has shed light on the inhibitory action of copper on kiwifruit infection by *Psa*. This newfound understanding of copper's inhibitory effect can contribute to the sustainable use of copper bactericides in agriculture. Furthermore, my research has confirmed that prolonged exposure to copper can lead to the emergence of copper-resistant pseudomonad strains. Interestingly, I found that resistance to aminoglycosides appears to co-evolve with copper resistance, particularly in strains derived from *P. fluorescens* SBW25.

My work also provides insights into the potential roles of two-component regulatory systems (CopRS and EnvZ-OmpR) and transcriptional regulators (HutC and KefA). They may act as global regulators in copper resistance, antibiotic resistance, and *Psa* virulence. This understanding could pave the way for new strategies in managing bacterial diseases and promoting sustainable agriculture.

Contents

Introduction

Utilization of copper (Cu) is important for all life forms. The two redox states of copper, Cu^{+} and Cu^{2+}, allow metalloenzymes to maintain normal biochemical processes (Osredkar, 2011). However, copper ions can induce oxidative stress when accumulated, and this can be lethal to cells (Cuypers et al., 2010; Kawakami et al., 2008). Given these properties, copper ions have been harnessed in commercial copper-based bactericides for the control of bacterial pathogens that cause crop diseases in the agricultural sector. Although copper-based bactericides are one of the most effective tools available for the control of bacterial crop pathogens, their mode of action is unclear. Importantly, the extensive usage of copper leads to phytotoxicity in plants (Lamichhane et al., 2018), reduces soil microbial biodiversity (Deng et al., 2009), and promotes the evolution of copper resistance in pathogenic bacterial strains (Colombi et al., 2017; Marin et al., 2019). The emergence of copper-resistant bacterial strains raises the possibility that, in the near future, we might lack competent ways of controlling these plant pathogens.

Many studies have found that bacterial strains with increased copper resistance also exhibit increased resistance to antibiotics (Alonso et al., 2001; Baker-Austin et al., 2006; Summers, 2002), suggesting that copper and antibiotic resistance frequently evolve together because of the pleiotropic effects of copper resistance. Thus, the continued application of copper-based bactericides in the agricultural sector could select for pathogens with resistance to both copper- and antibiotic-based antimicrobial agents, a worst-case scenario. This would lead to a loss of control for crop diseases and reduced crop production. Thus, having a deep understanding of the potential mechanistic links between copper resistance, antibiotic resistance, and bacterial virulence is critical.

The practical use of copper in agriculture.

Mechanisms of copper toxicity in bacteria.

Copper (Cu) is an essential trace element for all life forms (Finney & O'Halloran, 2003). Through a redox reaction, this metal, in its ionic form, can swap between two different states: the more biologically inert Cu^{2+} ion (the oxidized form), and the Cu^{+} ion (the reduced form). This characteristic allows this metal to act as a co-factor for metalloenzymes that are important for cell metabolism (Osredkar, 2011). Under normal conditions, an equilibrium of copper ions is maintained in cells. However, this balance

can be broken when cells are exposed to a high-copper environment. This causes an accumulation of copper ions, which is highly toxic, as it results in the production of reactive oxygen species (ROS, OH·) (Cuypers et al., 2010; Kawakami et al., 2008; Osredkar, 2011) through the Fenton-like reaction and Haber-Weiss cycle (Cuypers et al., 2010; Liochev & Fridovich, 2013).

The Fenton-like reactions is:

$Cu^{+}+H_2O_2 \rightarrow Cu^{2+}+OH\cdot+OH^{-}$

The Haber-Weiss reaction is:

$O^{2-}+H_2O_2 \rightarrow O2+OH\cdot+OH^{-}$

Copper ions can also deplete sulfhydryl from amino acids, cysteine and methionine, to generate hydrogen peroxide (H_2O_2). This molecule will then enter the above-mentioned reactions to assist with the amplification of free ROSs, which can inhibit cellular respiration and cause irreversible oxidative damage to cell components, including proteins and deoxyribonucleic acid (DNA) (Zhao & Drlica, 2014). This is also commonly known as oxidative stress. Copper-induced oxidative stress was documented in *Escherichia coli* (Gram-negative bacterium) and *Staphylococcus aureus* (Gram-positive bacterium) (Díaz-García et al., 2019).

A non-Fenton copper-induced toxicity, on the contrary, was identified in *E. coli* (Vincent et al., 2018). The accumulation of copper ions can displace the iron atom (Fe) from the iron-sulphur cluster enzyme involved in DNA synthesis and dioxygen processes (Brzóska et al., 2006; Kawakami et al., 2008). This displacement of the iron atom interferes the electron transfer pathways and leads to cell apoptosis.

These multisite and board-spectrum antimicrobial properties of copper ions make this heavy metal an ideal component for bactericides, fungicides, algaecides, and other antimicrobial agents utilized in public health and agriculture.

The history of copper-based antimicrobial product development.

The antimicrobial characteristics of copper have been known since 2600 BC, and it has been widely used for controlling diseases throughout human civilization. For example, copper

cooking tools were used in the Roman Empire (Borkow & Gabbay, 2009) and it was used for treating a variety of diseases such as cholera and lupus in the early 19th century (Grass et al., 2011). Even though there is a long history of humans using copper in medicine, the use of this metal in agriculture only started in the late 19th century when the Bordeaux mixture, a bactericide composed of copper sulphate and copper oxide, was accidentally identified by Millardet (Lamichhane et al., 2018). Since then, copper-based compounds have been wildly applied in the agricultural sector to manage diseases, having revolutionary effects on crop protection. Recently, many active agrochemicals- based on different copper compounds – have been implemented in agriculture (Table 1.1) (Athanassiou et al., 2018; TORRE et al., 2018).

Table 1.1: A list of copper compounds commonly used in agriculture.

Copper compound	Chemical formular
Copper sulphate	$CuSO_4 \times 3Cu(OH)_2$
Copper carbonate	$CuCO \times 3Cu(OH)_2$
Copper hydroxide	$Cu(OH)_2$
Copper oxide	Cu_2O
Copper oxychloride	$3Cu(OH)_2CuCl_2$
Copper oxychloride sulphate	$Cu_4(OH) \times 6(SO_4)$
Copper sulphate pentahydrate	$CuSO_4 \times 5H_2O$

Application of copper-based bactericides in crop protection.

With the rapid growth of the global human population, the field of agriculture is facing several challenges to meet the needs of feeding a significant number of people. One of the most important challenges is to prevent crop losses caused by pathogens, which are estimated to be 20 to 30% annually, equivalent to $220 billion USD (Rizzo et al., 2021). Thus, crop disease management is becoming a research priority in the agricultural sector.

The significant and broad-spectrum activities of copper against bacteria, along with its low toxicity to plants and mammals, make this metal element an effective agent for the control of bacterial pathogens associated with diseases that cause significant crop losses. Commercial copper-based products have been used for minimizing bacterial spot in tomato (Varympopi et al., 2022), zebra chip in potatoes (Mora et al., 2021), bacterial

cankers in citrus fruits (Behlau et al., 2017), and diseases in many other plants. These products are also commonly used to control *Pseudomonas syringae* pv. *actinidiae* (*Psa*, Gram-negative bacterium), which causes bacterial canker of kiwifruit, an important fruit for the economy of New Zealand.

The outbreak of *P. syringae* pv. *actinidiae* (*Psa*) in New Zealand.

Actinidia chinensis (kiwifruit) is a very nutritious fruit and was New Zealand's largest horticultural export in 2021 (Turner & Research, 2021). According to Horticulture New Zealand, kiwifruit exports were valued at $2,709 million in 2021, accounting for 43.7% of total fresh fruit export (Turner & Research, 2021). However, the kiwifruit industry was seriously threatened by a sudden outbreak of *Psa* in 2010 (Everett et al., 2011; Vanneste, 2017). The spread of this pathogen was quick and extensive after its introduction to New Zealand through transition of contaminated crops (Figure 1.1), causing an estimate of $900 million lost in revenue over the next 2 years (Birnie & Livesey, 2014).

The outbreak of *Psa* also brought a fundamental change in kiwifruit planting among commercial growers. The traditional gold-kiwifruit cultivar "Hort16A" was found to be highly susceptible to *Psa*, which was the main loss during the outbreak. Hence, a new *Psa*-tolerant cultivar, "Gold 3" (G3), was released to the market and immediately went into full-scale commercial production to replace Hort16A (Plant and Food Research, 2019). Nonetheless, the potential threat of *Psa* persists.

After a decade of efforts in controlling *Psa* infection and transmission, no competent ways to eliminate the bacteria from New Zealand's kiwifruit orchards have been found. Therefore, researchers have focused their attention back on copper-based bactericides.

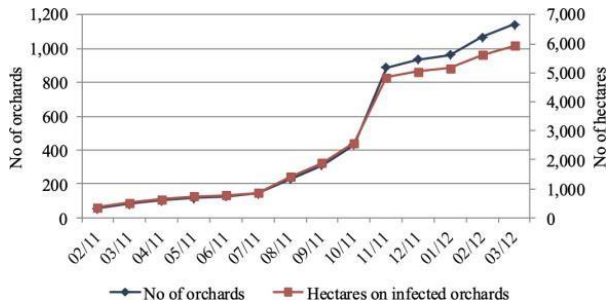


Figure 1.1: The spread of *Psa* in New Zealand kiwifruit orchards (Greer & Saunder, 2012), Graph indicating the change in total number of orchards and hectares of land infected by *Psa* between 02/2011 and 03/2012.

The use of commercial copper-based products in kiwifruit orchards.

Copper-based bactericides can significantly reduce the formation of bacterial cankers on kiwifruit vines, and thus it is considered as the most effective way of controlling *Psa* infection in New Zealand when there is no possibility to completely remove *Psa* from the orchards (Vanneste et al., 2011). Although suitable timings for bactericide treatment have been reported, the optimal application rates have not been generally agreed (Jeyakumar et al., 2014). An annually reviewed guideline, which is based on ongoing research, for the usage of copper-based bactericides is provided to growers from the Kiwifruit Vine Health (KVH) group and Zespri to prevent future loss of kiwifruit due to *Psa* (Table 1.2).

Table 1.2: Commercial copper-based bactericides recommended by KVH (2020-2021).

Bactericide product name	Copper formular	KVH recommended usage
Blue Shield DF	$Cu(OH)_2$	150-200g/100L
Kocide® Opti™	$Cu(OH)_2$	70-90g/100L
Nordox™ 75WG	CuO	37.5g/100L (summer),55-70g/100L (winter)
Tri-based Blue®	Tribasic $CuSO_4$	150ml/100L
Yates Copper Oxychloride	$Cu_2(OH)_3Cl$	500g/100L
Yates Liquid Copper	$C_4H_{10}CuNO_4$	300-750ml/100L, 25L/100L (stem cankers)

Potential risks associated with extensive use of copper-based agrochemicals.

The extensive use of copper-based agrochemicals can result in direct and indirect negative impacts on plants and the environment. This includes phytotoxicity to crop plants, reduced soil biodiversity, and the evolution of copper-resistant bacterial strains.

Phytotoxicity to plants

Copper ions continuously released from bactericides can accumulate in agricultural land and on plant surfaces, which can stress out plants and result in phytotoxicity that reduces crop yield and quality.

The phytotoxic effects of copper ions have been reported in tomatoes, apples, cherries, citrus, and pears (Lamichhane et al., 2018). However, studies conducted to investigate copper-induced phytotoxicity in kiwifruit are rare. To the best of my knowledge, only one study has investigated the phytotoxicity impacts of three commercial products

(Kocide™, Champ™, and Nordox™) in kiwifruit orchards (Jeyakumar et al., 2014). This study suggested that there was a light to moderate level of phytotoxicity on copper-treated individuals, but none of them showed severe symptoms. Thus, copper spraying remains the most suitable practice for *Psa* management.

Reduction in soil biodiversity

The accumulation of copper ions in the soil has been observed in copper-treated agricultural land in New Zealand. For example, the concentration of copper ions in the soil of a kiwifruit orchard in the Bay of Plenty region was monitored between 2009 to 2012. They reported that there was a significant increase of copper concentration in the soil since the 1st application of copper spray in 2009 (Guinto et al., 2012). This is potentially harmful to the soil micro-biota. Previous studies have shown that the microbial biomass, diversity, and enzyme activities in similar soil conditions were reduced (Figure 1.2) (Kandeler et al., 1996; Naveed et al., 2014). Furthermore, shifting in the microbial community structure is prevalent in copper-contaminated soil (Deng et al., 2009).

The shrinkage and modification of soil biodiversity can restructure chemical and biological compositions, and thus affect soil health. Over the long term, this can lead to challenges with crop production.

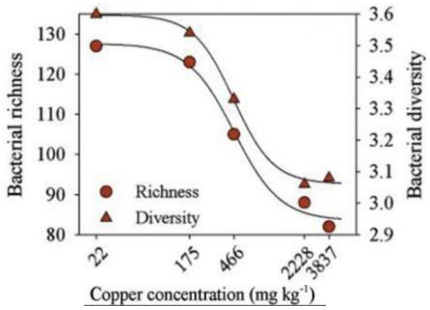


Figure 1.2: Richness and diversity of soil bacteria in response to soil copper concentration(Naveed et al., 2014)**.** The diversity and richness of the microbial community were studied in copper- contaminated soil.

Emergence of copper-resistant strains

The copper-rich environment on plant surfaces and in soil promotes the emergence of copper-resistant (Cu^R) bacterial strains. Characters of Cu^R strains that can grow in high-copper habitats were reported for *Xanthomanoas citri*, the causative agent of bacterial canker in citrus, which is commonly controlled by copper spraying (Figure 1.3) (Marin et al., 2019). The ability of these Cu^R strains to survive and multiply under the standard

practise of copper treatment reduces the effectiveness of the management strategy, and likely contributes to the loss of canker disease control in citrus orchards.

Cu^R *Psa* strains were isolated from New Zealand's kiwifruit orchards after four years of copper spraying in 2014. These strains, *Psa* NZ45 and *Psa* NZ47, which originate from a strain that lacks copper resistance genes, NZ13 (Colombi et al., 2017), were isolated from two geographically distinct orchards (McCann et al., 2013). The identification of these strains raised a concern regarding the molecular and evolutionary responses of *Psa* to copper bactericides. With this concern in mind, it is vital we understand the mechanisms underlying these responses in *Psa*.

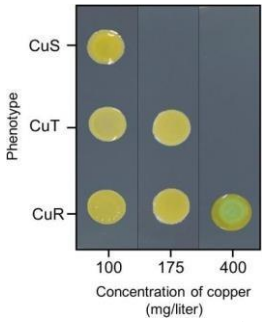


Figure 1.3: Characterization of copper-resistant/tolerant *Xanthomonas citri*strains in copper environments (Marin etal., 2019)**.** Copper-sensitive (CuS), copper-tolerant (CuT), and copper- resistant (Cu^R) *X. citri* strains were grownon mannitol-glutamate-yeast extract (MGY) agar plates supplemented with varying concentrations of Cu^+.

Molecular mechanisms of copper resistance in Gram-negative bacteria.

Copper is critical for normal cellular metabolism; however, it is also extremely toxic when in excess in the cytosol. Therefore, bacteria have mechanisms to tightly control the level of copper ions within cells. The principle of copper resistance in bacterial cells is based on: **1)** the extracellular and intracellular sequestration of Cu^+ by cysteine-rich metallothionein proteins; **2)** the detoxification of Cu^+ to less toxic Cu^{2+} ions via multicopper oxidases; and **3)** the active export of $Cu^{+/2+}$ from the intracellular to extracellular space through efflux pumps. These mechanisms enable bacteria survival in high copper situations.

As Gram-negative bacteria have an extra cell membrane, they must manage the concentration of copper ions not only in the cytoplasmic environment, but also the

periplasmic environment. In the following sub-sections, some well-studied molecular mechanisms involved in copper resistance will be described using Gram-negative bacteria as model organisms.

Cue system

Cue system as the primary resistance mechanism against copper in E. coli.

The cue (Cu efflux) system acts as the primary and the main mechanism for Cu^R in *E. coli* (Rensing & Grass, 2003). Three proteins cooperate with each other to maintain a low copper concentration in the cell. **1) CueR**, a copper-responsive Mer-R-like metalloregulator, binds to the inverted-repeated sequence (ACCTTCC-N7-GGAAGGT) of cue promotors to regulate the expression of *cueA* and *cueO* (Fang et al., 2021; Grass & Rensing, 2001); **2) CueA**, a copper-transporting P-type ATPase, translocate Cu^+ ions from the cytoplasm to the periplasm (Bondarczuk & Piotrowska-Seget, 2013); and **3) CueO**, a periplasmic multicopper oxidase oxidizes Cu^+ to Cu^{2+} and reduces dioxygen (O2) in water molecules (Figure 1.4) (Djoko et al., 2010).

The detoxification efficiency of Cu^+ ions via multicopper oxidases is highly dependent on the availability of copper-binding sites in the methionine-rich helix in its structure. A typical multicopper oxidase comprises three types of binding sites: Type 1 (T1) is folded in the center of the protein to oxidize Cu^+ ions, while Type 2 (T2) and Type 3 (T3) form a trinuclear center to reduce oxygen (Roberts et al., 2002). CueO in *E. coli* has an additional methionine-rich helix in T1 binding sites, which significantly enhances its Cu^+-oxidizing ability (Dupont et al., 2011). A similar methionine-helix structure was also identified in PcoA of *E. coli* and CopA in *P. syringae* (Roberts et al., 2002).

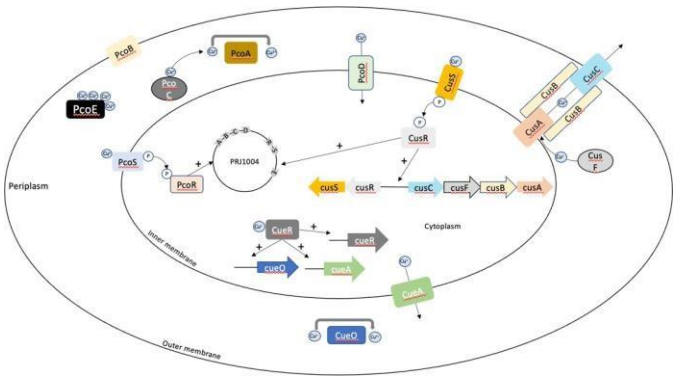


Figure 1.4: Illustration of the molecular mechanisms of copper resistance in *E. coli*. Three systems –the Cue, Cus and Pco systems – are shown in this diagram. When CusS and PcoS sense the change of $Cu^{+/2+}$ level, they phosphorylate their complemented partners (CusR and PcoR). CusR, PcoR and CueR activate the transcriptional process of their downstream genes encoding for a P-type ATPase (CueA), multicopper oxidases (CueO, PcoA), and copper chaperons (CusF, PcoC), etc. These proteins either detoxify Cu^+ ions to less toxic Cu^{2+} ions,

export excess $Cu^{+/2+}$ ions out of the cell or sequestrate $Cu^{+/2+}$ ions to prevent excess copper inside the cell. In combination, the three systems form a network for fully functional copper resistance in *E. coli*.

The Cue system in Pseudomonas.

Pseudomonas demonstrates varying metabolic diversity and can colonize many diverse niches. For example, *P. aeruginosa*, a human pathogen, is often responsible for opportunistic infections in healthcare settings, while several *P. syringae* pathovars infect stone fruits, resulting in bacterial cankers, and *P. fluorescens*, a plant-promoting bacterium, frequently colonizes the plant root system. Like *E. coli*, the *Pseudomonas* genus has evolved with a relatively well-studied Cue system to support their survival in copper-rich environments.

The CueR-like transcriptional regulator commonly exists in Gram-negative bacteria and has been studied in detail in *Pseudomonas*. Upon copper addition, CueR activates the expression of the copper chaperon genes *cueA* and *cueZ*, and autoregulates itself in *P. aeruginosa*, *P. putida* and *P. fluorescens* (Adaikkalam & Swarup, 2002; Thaden et al., 2010; Zhang & Rainey, 2008). The expression of *cueR* was found to be regulated by a global regulator, LasR, which is well-known for its regulatory role in the expression of virulence genes in *P. aeruginosa* (Schwan et al., 2005). This is strong evidence that copper resistance could be linked to bacterial pathology.

The *cueA* operon encodes for the CopA1 P-type ATPase in *P. fluorescens* as well as another copper-ATPase called CopA2. Both are induced not only by high copper concentration but also upon direct contact of the bacterium with plants (Rademacher & Masepohl, 2012). This indicates that these energy-dependent enzymes play important roles in bacterial-plant associations. Interestingly, CopA2 in *P. aeruginosa* is not essential for copper tolerance, even though it shows capability in exporting Cu^{+} out of cytosol (González-Guerrero et al., 2010). Together, up-to-date evidence suggests that *cue* genes in *Pseudomonas* may have dual functions in copper resistance and bacterial- host interactions.

Cus system

The Cus system is another well-studied mechanism responsible for maintaining a low level of Cu^{2+} in *E. coli* cells. Unlike the Cue system, which requires O_2 as a reduction

substrate, the CBA-type efflux pump in the Cus system can operate under anaerobic conditions (Outten et al., 2001). The CBA-like pump is usually made up of three components: **the resistance-nodulation-cell division superfamily (RND)**, which is embedded in the inner membrane and is responsible for translocating substrate from the cytosol; **the outer membrane factor family (OMF)**, which forms a channel to export substrate to the extracellular space; and **the membrane fusion protein family (MFP)**, which enable interactions between RND and OMF in the periplasmic space (Kim et al., 2010).

The CusCFBA pump in *E. coli*.

In *E. coli*, a tetrapartite CBA-like pump named CusCFBA is involved in copper resistance (Figure 1.4). This efflux pump has also been found to participate in bacterial silver tolerance (Franke et al., 2003; Grass & Rensing, 2001). The **CusA subunit** of the CusCFBA protein, which belongs to the RND family, is formed by three homotrimers (12 transmembrane monomers each) in the inner membrane (Long et al., 2010). This inner membrane protein transfers Cu^+ ions from the cytoplasm to the periplasm (Su et al., 2011). On the other hand, the **CusC subunit**, which is a member of the OMF family, is embedded in the outer membrane and forms a negatively charged channel that exports Cu^+ ions outside of the cell (Delmar et al., 2013). The two membrane-embedded subunits are connected by the funnel-structured **CusB subunit**, which acts as a bridge to transport Cu^+ ions across the periplasmic space (Delmar et al., 2013). The last component in the CusCFBA system is **CusF**. It is folded into a small metallochaperone that is responsible for the sequestration and transfer of Cu^+ ions into the CusCBA pump in the periplasm (Bagai et al., 2008).

A tight control of expression on *cus* genes is achieved through a two-component regulatory system, **CusRS**. The inner membrane receptor, CusS, can sense the elevation of copper or silver ions and activate CusR by phosphorylation (Gudipaty et al., 2012). CusR then binds to an operon binding site (AAAATGACAA-N2-TTGTCATTTT) of the *cusC* and *cusR* promotors to tigger the transcription of the *cus* gene clusters (Bondarczuk & Piotrowska-Seget, 2013). In addition to the two-component regulatory systems, two other systems (CpxRA and YedWV) are involved in a copper-induced manner. The CpxRA system can detect copper-induced misfolded proteins and regulates genes involved in envelope stress responses, while the YedWV system recognises an increased level of H_2O_2 in the cell and controls the same targets as the CusRS system (Urano et al., 2015). These systems collaborate to enhance copper resistance responses in bacteria

to reinforce survival rate in copper-rich habitats.

cus-like genes in *Pseudomonas*.

A specific Cus system has not yet been identified in *Pseudomonas* species. A set of gene loci (*PA3521*, *PA3522*, and *PA3523*) in *P. aeruginosa* were predicted as *cusC*, *cusA*, and *cusB* genes, respectively, based on their sequences (Quintana et al., 2017); but these genes have been reported to encode a multidrug efflux pump, MexPQ-OpmE, in an earlier publication (Mima et al., 2005). The bioinformatic analysis as a sole piece of evidence is not strong enough to claim that these genes encode for Cus proteins, so it is generally agreed that *Pseudomonas*-specific *cus* genes have not yet been found.

Nonetheless, a set of genes, *czcCBA*, reported to encode a heavy metal (Co^{2+}, Zn^{2+}, and Cd^{2+}) transporter, have been described in *P. aeruginosa* (Nies, 1995). A comparison study has indicated that *czcCBA* are phylogenetically close to *E. coli* CusCBA on protein level (Mima et al., 2005); however, the deletion of *czcA* had no impacts on bacterial copper sensitivity, indicating that the CzcCBA transporter may not be involved in copper resistance (Teitzel et al., 2006). There are no further research papers focusing on *cus* genes in *Pseudomonas* and the conservation of "Cus" proteins between *Pseudomonas* and the model organism *E. coli*, at the amino acid level, is low. It is highly likely that *Pseudomonas* species do not rely on the Cus system for copper regulation.

Plasmid-encoded copper resistance elements

Plasmid-encoded resistance genes are commonly found in bacteria and are used as the main strategy of bacteria to gain additional functions of protection against antimicrobial substances in their surroundings. The existence of these plasmids also facilitated the evolutionary process of bacterial resistance to copper. Thus, understanding what copper resistance systems are encoded by in plasmids is critical for us to study evolution.

Pco system in *E. coli*.

The Pco system is also known as the plasmid-born copper resistance system (Figure 1.4). It includes seven genes, *pcoABCDRSE*, which are all are under the control of a copper-induced promoter on the pRJ1004 conjugative plasmid in *E. coli* (Huffman et

al., 2002). **PcoA** is a multicopper oxidase that functionally substitutes but is genetically distant from, CueO (Lee et al., 2002). **PcoB** is an outer membrane protein that acts as a guard to prevent Cu^+ uptake from the external environment (Rensing & Grass, 2003). Like other metallochaperones, **PcoC** is a small methionine-rich protein responsible for sequestration of Cu^+ ions and bringing them to PcoA for exportation (Lee et al., 2002). Interestingly, **PcoD** is a copper importer that maintains copper homeostasis through a mechanism of Cu^+ ion uptake rather than export (Lee et al., 2002).

A two-component regulatory system, **PcoRS**, governs the expression of *pco* genes. PcoS is a signal transduction sensor that activates PcoR for regulation of all downstream *pco* genes (Figure 1.4) except *pcoE* (Brown et al., 1995). **PcoE**, a chaperon, is highly induced by elevated copper concentration and it is under the control of CusRS (Munson et al., 2000). This chaperon is predicted to be an initial player for Cu^+ ion sequestration in the periplasm before the Pco system is ready (Lee et al., 2002). These proteins together form the operative mechanism to balance intra-and-extracellular Cu^+ ions in *E. coli*.

Cop system in *Pseudomonas*.

The plasmid-encoded Cop system in *Pseudomonas* species is a homologue of the Pco system in *E. coli*. It was first identified in the plant pathogen *P. syringae* pv. *tomato*. The system consists of structural genes (***copABCD***) and regulon genes (***copRS***), all of which are encoded by the pPT23D plasmid in *P. syringae* pv. *tomato* (Cha & Cooksey, 1991).

The main component of the system is the **CopA** protein, which belongs to the multicopper oxidase family and has high a capacity for Cu^+, binding 11 Cu^+ ions at once (Silver, 1996). The second participant, **CopB**, is embedded on the outer membrane and binds Cu^+ ions to avoid excess absorption via passive transport (Puig et al., 2002). **CopC**, on the other hand, act as a metallochaperone that delivers Cu^+ ions to CopA, while **CopD** is an inner membrane protein that imports essential Cu^+ ions to maintain copper homeostasis (Arnesano et al., 2002). The genes encoding these proteins are regulated by the **CopRS** two-component regulatory system, with *CopS* and *CopR* continuously transcribed to sense changes in the intracellular Cu^+ concentration and activate *cop* genes respectively (Puig et al., 2002).

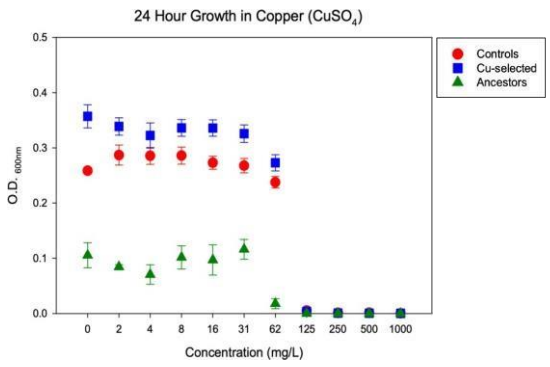
The evolution of copper resistance in bacteria.

Bacteria can respond to habitat changes and adapt to adverse conditions. This ability is defined as resistance. Generally, adaptation to the environment depends on physiological changes in response to specific signals via the regulation of gene expression (Rodríguez-Verdugo et al., 2016). However, bacteria can gain the function of antimicrobial resistance through modification of genes at chromosomal level (DNA mutations) and might receive additional utility via horizontal gene transfer (HGT) of mobile genetic elements including plasmid and integrative conjugation elements (ICEs). Together, these events can enhance bacterial resistance to copper if copper-resistance genes are involved. It has been reported that copper loses its effectiveness in bacterial control in agriculture due to the emergence of copper-resistant strains. In the following subsections, the two known mechanisms of evolution for copper resistance will be briefly described with examples.

Evolution of copper resistance via mutation

Mutation is a change in the DNA sequence through deletion, insertion or substitution of nucleic acid bases. This can lead to a shift or alteration of DNA sequences that affect protein production or sequence. In this way, these changes can result in detectable phenotypic changes for an organism. The exposure to extreme environment can be a trigger of genetic mutation.

Recently, an evolutionary study on *E. coli* successfully established a significant increase in Cu^R in evolved strains (Figure 1.5). In this study, 11 mutations in the *cpxA*, *cpxR*, or *cpxP* genes were identified across seven of nine evolved strains (Boyd et al., 2022). The CpxRA signal transduction system is activated by Cu toxicity (Yamamoto & Ishihama, 2006) and is known to participate in controlling the *scsABCD* operon, with the products of



this operon counteracting copper stress (López et al., 2018). A common mutation in *cpxA* and *cpxR* across evolved strains suggested that they have adaptative importance to *E. coli* in copper-rich surroundings. This is also strong evidence showing antimicrobial properties can be gained through mutations.

Figure 1.5: Illustration comparing the copper resistance of Cu- selective strains to the ancestors (Boyd et al., 2022)**.** The population density of Cu-selected and ancestor *E. coli* strains are compared in response to varying concentration of $CuSO_4$.

Evolution of copper resistance via horizontal gene transfer

The evolution of copper resistance through HGT has been studied in many bacteria. The spread of function relating to antimicrobial resistance in a bacterial community via acquisition of a plasmid has been relatively well established; however, the evolution of resistance via ICEs is not well understood, especially in *Pseudomonas* species.

In another study, the genomic sequences of *copRS*, *cusABC*, and *copACBD* in copper-resistant *Psa* strain, NZ45, were compared with two ICEs from *P. syringae* pv. *atrofasciens* and *P. syringae* pv. *panici*; and a plasmid from *Psa* J2 (Figure 1.6) (Colombi et al., 2017). The comparison suggested that Cu^R genes of NZ45 are recombinants and originated from the abovementioned species. Considering these species are phylogenetically distinct, the paper claims NZ45 acquired these ICEs through HGT by conjugation (Colombi et al., 2017).

Together, bacteria have a good chance of evolving higher Cu^R through different mechanisms, but genetic regulation is a network, meaning that alterations in one base pair could have significant impacts on another, especially when global regulators are involved. In this case, two or more phenotypes could be modified at once and this is known as co-evolution.

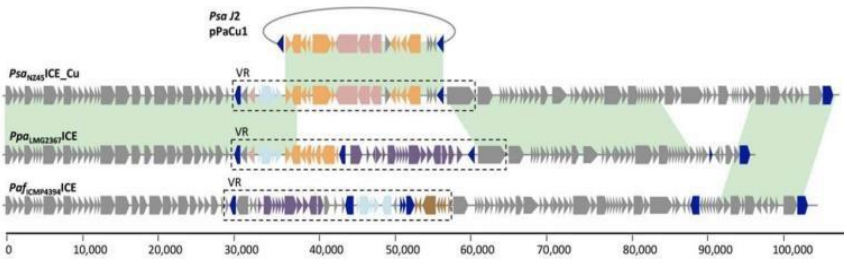


Figure 1.6: Genetic organization of Cu^R ICEs in *Psa* NZ45 (Colombi et al., 2017). Blue boxes are mobile genes; purple boxes are "enolases"; orange boxes are copper resistance genes; and pink boxes are *cus* genes. Areas covered with green indicate over 99% at nucleotide level.

The potential co-evolution of copper resistance, antibiotic resistance, and bacterial virulence.

The toxicity of agriculturally used antibiotics.

Antibiotics are antimicrobial agents that can inhibit bacterial growth or directly kill bacteria. The first antibiotic identified was penicillin, which is widely use in medication in human civilization. The powerful competence of antibiotics to Cu^R and save patients' lives immediately raised interest among plant pathologists. Since the 1950s, more than 40 antibiotics have been screened for crop protection and have been used until today (McManus et al., 2002).

Aminoglycosides are one of the oldest antibiotics derived from actinomycetes. Their antimicrobial activities are board-spectrum and are the result of them binding to the acceptor-site (A-site) of 16S ribosomal RNA to inhibit protein synthesis (Kotra et al., 2000). Tobramycin, gentamycin, and streptomycin are examples from this family. Among them, gentamycin and streptomycin are commonly used for manging fire blight in apples and pears, or diseases cause by *Agrobacterium*, *Erwinia*, *Pseudomonas*, and *Xanthomonas* (McManus et al., 2002).

Tetracyclines were discovered in the 1940s and exhibit low toxicity but high inhibition effects against both Gram-negative and Gram-positive bacteria. The mode of action for tetracyclines is through blocking the A-site on the 30s ribosome, which again results in protein synthesis inhibition (Chopra & Roberts, 2001). In agriculture, the application of oxytetracycline is a standard practice for controlling vegetable diseases caused by *Erwinia*, *Pectobacterium*, and *Pseudomonas* etc. (McManus et al., 2002).

Unlike the two antibiotic families mentioned above, which can both be purified from living organisms, quinolones are synthetic antibiotics can be isolated from chloroquine. Antibiotics from this family directly inhibit DNA replication by binding to topoisomerase IV or DNA-gyrase-DNA complexes, to reduce bacterial multiplication (Hawkey, 2003). Members from this family include ciprofloxacin, nalidixic acid, and oxalinic acids.

The rapidly elevating needs on crop production to feed the population, along with the spread of disease due to anthropologic activity, have dramatically enhanced the use of antibiotics to prevent production loss. According to the National Agriculture Statistics Survey (NASS), up to 65,227 kg of streptomycin and oxytetracycline are applied to farmland each year in the USA (McManus et al., 2002). However, the extensive use of antibiotics has promoted the emergence of antibiotic-resistant strains, which brings negative impacts on the effectiveness of antibiotics in disease control.

The evolution of streptomycin-resistant (Sm^R) bacterial pathogens has led to failures in disease management and is well-documented. Sm^R *X. campestris* pv. *vesicatoria*, for example, has reduced the effectiveness of streptomycin in controlling the infection by this bacterial pathogen in pepper and tomato in the USA (Ritchie & Dittapongpitch, 1991). Similar outcomes were also reported for *P. carotovara* (responsible for angular leaf spots in cucumber) (FUKASAWA et al., 1980), *P. chichorii* (bacterial blight in celery) (Pohronezny, 1994), *P. syringae* pv. *papulans* (blister spot in apples) (Jones et al., 1991), and *P. syringae* pv. *syringae* (blossom blasts in apples) (Huang & Burr, 1999).

These real-life examples have shown the risk of large-scale usage for not just antibiotics but also the substitute, copper, if uncontrolled application of antimicrobial agents continues. What concerns the field most is that there seems to be a co-evolutionary trend between copper and antibiotic resistances, meaning it is possible that the usage of one antimicrobial agent could also weaken the other agent's effectiveness.

The underlying mechanisms of co-evolution between copper and antibiotic resistance.

The extensive application of antibiotics in farmland settings has stimulated the proliferation of antibiotic resistance in bacterial populations, while many studies have advised that Cu^+ ions and other heavy metals in the environment also play critical roles in this phenomenon (Alonso et al., 2001; Summers, 2002). Anthropogenic metal sources, such as through copper spraying in agriculture, are the major cause of heavy metal contamination; these sources can lead to the development of metal resistance in bacterial populations and induce the in-direct co-evolution of antibiotic resistance (Baker-Austin et al., 2006).

Interestingly, the mechanisms of antibiotic and heavy metal resistance share similar characteristics. The principal mechanisms are exportation, detoxification, inactivation, and reduction of uptake (Cox & Wright, 2013). The highly overlapping systems indicate that the underlying mechanism of co-evolution could be cross-resistance (i.e., a single physiological mechanism confers antibiotic and metal resistance) (Seiler & Berendonk, 2012). The cross-resistance between copper and β-lactam resistance via the AcrD and MdtABC efflux pumps under the regulation of the BaeSR two-component regulatory system was documented in *Salmonella typhimurium* (Nishino et al., 2007).

In the worse-case scenario, the application of copper across the agricultural sector could select for bacterial strains that have overlapping resistances to copper and antibiotics. If pathogenic are species involved, this would promote the emergence of highly virulent strains with high levels of resistance to antimicrobial agents, ultimately leading to a lack of effective practices for crop disease control. Several studies have reported the identification of antibiotic-resistant bacterial strains in copper-contaminated soil (Berg et al., 2005; Hu et al., 2016). However, the potential and underlying mechanisms of dual resistance remain unclear.

Copper resistance and virulence in pathogens.

Copper is important for a functional immune system in hosts. Animal models with copper deficiency result in reduced production of antibodies (Prohaska & Lukasewycz, 1981), suppression of neutrophils, and a macrophage-based respiratory burst (Babu & Failla, 1990). In plant hosts, copper plays a role in the immune system via the ethylene (ET) biosynthesis pathway which was first identified in *Arabidopsis* (Y. Liu & Zhang, 2004). The ET pathway, for example, is activated by Cu^{+}-induced *StABA1* and *StNCED1* gene suppression, which are genes that are responsible for abscisic acid (ABA) production. This ET pathway activation ultimately results in immune responses active against bacteria and fungi (H. Liu et al., 2020).

In general, bacterial virulence is positively related to copper resistance. In other words, copper hypersensitivity (i.e., a reduction in copper resistance) attenuates bacterial virulence or vice versa. or instance, with relation to the mouse model, mutation of the *copA1* gene in *P. aeruginosa* decreases not only copper resistance, but also bacterial virulence (Schwan et al., 2005). However, this attenuation of bacterial virulence is

usually only apparent when the function of copper transporters is disrupted. Mutations in other copper-handling genes can also lead to opposite or no significant changes in virulence. For example, the *MmcO* (multicopper oxidase) deletion mutant has no effect on *M. tuberculosis* virulence (Rowland & Niederweis, 2012). The underlying reasons for the impacts on pathogenicity through different levels of copper sensitivity are unknown, and research focusing on plant pathogens in this topic is rare.

Overall, the molecular and evolutionary mechanisms of copper resistance in plant-associated bacteria determine how we go about managing crop diseases. Thus, improving our knowledge on these mechanisms is an immediate need for the agricultural sector.

Knowledge gaps, research aim and objectives.

Four knowledge gaps have been identified in this field:

1) The role of copper in inhibiting *Psa* infection on kiwifruit has not been studied systematically.

2) The evolutionary potential of copper resistance in *Pseudomonas* has rarely been studied.

3) The potential co-evolution of copper and antibiotic resistance is poorly understood.

4) The potential co-evolution of copper resistance and virulence in *Psa* is poorly understood.

With these knowledge gaps in mind, **the overall goal of this project is to improve our understanding of the molecular and evolutionary mechanisms of copper resistance in plant-associated bacteria.** The research work will focus on a model plant growth-promoting bacterium, *Pseudomonas fluorescens* SBW25, and two strains of the kiwifruit bacterial canker bacterium, *Pseudomonas syringae* pv. *actinidiae*.

To address the abovementioned knowledge gaps, three specific objectives were set for this work:

Objective 1: Determine the role of copper bactericides in inhibiting *P. syringae* pv. *actinidiae* (*Psa*) infection.

Copper-based bactericides will lose the effectiveness in controlling kiwifruit bacterial canker with the emergence of copper-resistant *Psa* strains and, thus, it is considered that the continuous use of copper will no longer provide further benefits in the field. It is commonly known that copper has the ability of inhibiting *Psa* infection on kiwifruit, but the roles of copper in this action have not been investigated. It is highly likely that copper bactericides can protect kiwifruit from *Psa* infection at non-inhibitory concentrations for *Psa* growth. To test this hypothesis, I will develop and validate a new experimental technique to quantify bacterial infection *in planta* (Task 1) and will use this new method to compare the inhibitory effects of copper bactericides on *Psa* growth versus infectivity on leaves (Task 2) (Figure 1.7).

Task 1: Develop and validate a new method for quantifying *Psa* infectivity on kiwifruit leaves.

This will be done by genetically engineering a set of bacterial strains so that bacterial activity can be determined through use of a bioluminescence reporter gene under the control of a constitutive promoter (P_{nptII}-*lux*). This allows the quantification of bacterial activity in the plant environment via measuring the "light" production which can be captured using a CCD camera.

Task 2: Quantifying the effects of copper on *Psa* infectivity.

The new technique developed in Task 1 will be used to determine the effects of bactericide concentrations on *Psa* infectivity and surface growth on kiwifruit leaves. The possible difference of *Psa* tolerance on cultivars (Hort16A and Hayward) will also be considered in this task.

By completing objective 1, I will have a reliable quantification method to examine pathogen infectivity and develop a better understanding of the potential differences between the inhibition effects of copper-based bactericides on *Psa* growth and infection. This will have significant impacts on sustainable use of bactericides in kiwifruit orchards.

Bioluminescence-labelling of *Psa* strains

↓

Develop the method of plant infection (Task 1)

↓

Examine the effects of copper bactericide on *Psa* infectivity in kiwifruit. (Task 2)

Figure 1.7: Outline for objective 1: investigating the effects of copper bactericides on *Psa* growth and infection. Bioluminescence-labelled *Psa* strains will be used as a bioreporter in this work. A novel method to quantify bacterial infection via measuring "light" expression will be developed and the technique used to examine the effects of copper bactericide on *Psa* infectivity in kiwifruit.

Objective 2: Experimental evolution of copper resistance in plant-growth promoting bacterium *P. fluorescens* SBW25.

To assess the evolutionary potential of copper resistance in *Pseudomonas*, a technique called colony-to-colony transfer was performed in previous projects (see Figure 1.8 and 1.9). This was done by growing bacterial cultures on gradient agar plates supplemented with $CuSO_4$, with single colonies growing on a relatively high concentration of $CuSO_4$ selected for the next transfer. Five replicate lines derived from SBW25 will be transferred 30 times and the final evolved strains will be subjected to genome re-sequencing (Task 1) and phenotypic assays (Task 2) (Figure 1.8) including their resistance to copper and antibiotics.

Task 1: Genome re-sequencing of the copper-evolved *P. fluorescens* strains.

The mutation accumulation work through colony-to-colony transfer was done by other students in previous project. Here, in this work, the genome of the final evolved strains will be re-sequenced and compared to the ancestral strain to identify for possible genes that might involve in copper and antibiotic resistance.

Task 2: Phenotypic characterization of SBW25 evolved strains.

Many field studies have shown copper-resistant isolates from agriculture lands also exhibit higher antibiotic resistance, indicating an underlying evolutionary mechanism is shared between copper and antibiotic resistance. In this work, I will characterize the change of degree of resistance to copper and different antibiotics (across different families) in evolved strains.

Results from objective 2 will reveal the evolutionary potential of copper resistance in the

beneficial bacterium *P. fluorescens* SBW25 and the potential correlation between copper and antibiotic resistance.

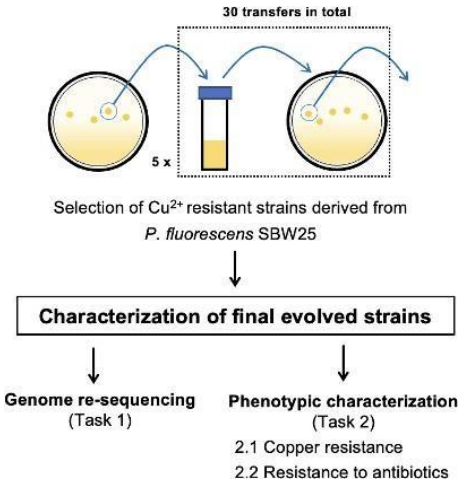


Figure 1.8: Outline for objective 2: experimental evolution of copper resistance in plant growth-promoting bacterium *P. fluorescens* SBW25. *P. fluorescens* SBW25 was subjected to colony-to-colony transfer on gradient agar plates with five replicate lines. After a total of 30 transfers, the final evolved strains will be characterized for resistance to ionic copper and antibiotics in this project. The genome of the evolved strains will also be re-sequenced to identify associated mutations in this work.

Objective 3: Experimental evolution of copper resistance in plant-pathogenic bacterium *P. syringae* pv. *actinidiae* (*Psa*).

To assess the potential relationship between copper resistance and bacterial virulence in *Psa*, two strains, NZ13 and NZ47, were subjected to colony-to-colony transfer (Figure 1.9) with 8 replicate lines transferred for ~20 times by a post-doc in a previous project. The final evolved strains will be subjected to genome re-sequencing (Task 1) and phenotypic assays (Task 2), and their resistance to copper and antibiotics will be assessed.

Task 1: Genome re-sequencing of the copper-evolved *Psa* strains.

The mutation accumulation work through colony-to-colony transfer was done by Dr. Naren in a previous project. Here, in this work, the genome of the final evolved strains will be re-sequenced and compared to the ancestral strain to identify genes that might be associated with copper resistance, antibiotic resistance, and virulence.

Task 2: Phenotypic characterization of *Psa* evolved strains.

Similar to objective 2, the phenotypes of investigation include copper and antibiotic resistance. On top of these, I will label the final evolved strains with the *lux* gene under the P_{nptII} promoter to investigate the bacterial virulence on kiwifruit plants with the new technique generated in objective 1.

Results from objective 3 will reveal the evolutionary potential of copper resistance in pathogenic *Pseudomonas* strains (*Psa* NZ13 and NZ47) and the potential correlation between copper and antibiotic resistance. More importantly, the data will be able to show the relationship between copper resistance and bacterial virulence in *Psa*, which has huge importance to the practice of controlling *Psa* in kiwifruit orchards in New Zealand.

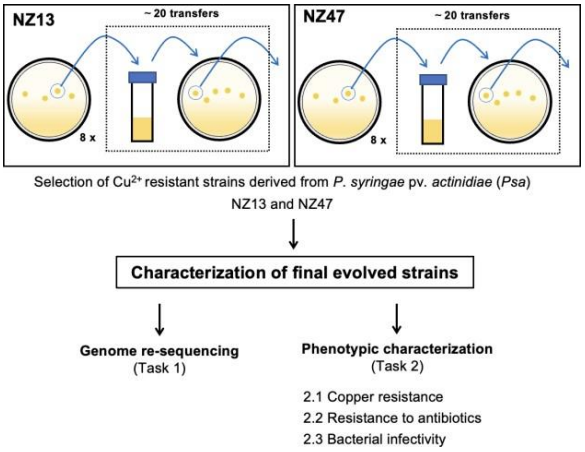


Figure 1.9: Outline for objective 3: experimental evolution of copper resistance in pathogenic bacterium *Psa*. *Psa* NZ13 and NZ47 were subjected to colony-to-colony transfer on gradient agar plates with eight replicate lines each. After a total of ~20 transfers the final evolved strains will be characterized with their resistance to ionic copper and antibiotics in this project. The final evolved strains will also be labelled with lux gene to test for their virulence with the novel method developed in objective 1. The genome of the evolved strains will also be re-sequenced to identify mutations in this work.

Materials and methods

Bacterial strains, plant cultivars, and growth conditions

Bacterial strains used in this study are listed in Table 2.1. The *P. fluorescens* SBW25 and *P. syringae* pv. *actinidiae* (*Psa*) strains were routinely grown in lysogeny broth (LB) and King's medium B (KB) medium, respectively. All bacterial strains were incubated at 28°C, unless specified. Strains were stored at -80°C for long-term storage and, thus, all strains were inoculated to appropriate media for activation before any experiments.

Actinidia chinensis (kiwifruit) cultivars of Hort16A and Hayward were used for plant-related experiments in this project. They were either purchased from Multiflower Laboratories as tissue culture plants (young plants grown in agar pots) or soil-grown mature plants. All plants were grown under well-controlled indoor conditions at 22°C and a 14/10 hr light to dark cycle with light intensity between 2000 to 6000 lux.

Table 2.1: Bacterial strains used in this work.

Strain name	Species, isolate	Evolutionary generation (number of transfers)	P_{nptII}-*lux*-labelled (Yes/No)
SBW25	*P. fluorescence*, SBW25	N/A, ancestor wild type	Yes
AP	*P. fluorescence*, SBW25	30	No
CP	*P. fluorescence*, SBW25	30	No
DP	*P. fluorescence*, SBW25	30	No
EP	*P. fluorescence*, SBW25	30	No
FP	*P. fluorescence*, SBW25	30	No
NZ13	*Psa*, NZ13	N/A, ancestor wild type	Yes
A1	*Psa*, NZ13	14	Yes
A2	*Psa*, NZ13	18	Yes
A3	*Psa*, NZ13	18	Yes
A4	*Psa*, NZ13	16	Yes
A5	*Psa*, NZ13	16	Yes
A6	*Psa*, NZ13	16	Yes
A7	*Psa*, NZ13	14	Yes
A8	*Psa*, NZ13	18	Yes
NZ47	*Psa*, NZ47	N/A, ancestor wild type	Yes
B1	*Psa*, NZ47	20	Yes
B2	*Psa*, NZ47	15	Yes
B3	*Psa*, NZ47	20	Yes
B4	*Psa*, NZ47	16	Yes
B5	*Psa*, NZ47	17	Yes
B6	*Psa*, NZ47	17	Yes
B7	*Psa*, NZ47	16	Yes
B8	*Psa*, NZ47	19	Yes

The generation of bioluminescence-labelled strains

Transformation via conjugation

All copper-derived *Psa* strains were labelled with pAG4-P_{nptII}-*lux* using a conjugation method. *E. coli* DH5a strains (containing the pRK2013 or pUX-Bl13 helper plasmids), the *E. coli* TOP 10 strain (containing the pAG4-P_{nptII}-*lux* plasmid) and the recipient *Psa* strain were prepared in LB for overnight cultures. Bacterial cells were collected by centrifugation at 4000g for 10 mins. Cultures were then washed with 0.9% NaCl and resuspended in 250 ul of 10 mM $MgCl_2$. All four bacterial strains were then mixed and grown on LB agar plates at 22°C for 24 hrs.

Bacteria were scrapped with a bent pipette tip from agar plates and washed into 1 ml of 10 mM $MgCl_2$. Culture was then spread on KB agar plate with 50 uM nitrofuran (NF) antibiotics and 5 uM gentamycin and incubate at 22°C for 72hrs for selection.

Strain validation by PCR

Polymerase chain reaction (PCR) experiments were used to validate the *lux*-labelling of *Psa* evolved strains after transformation and selection. Gene fragments were amplified by PCR from labelled *Psa* strain genomes using a *Psa*-specific primer, PsaglmS (3'-TCCACCAAGGCCTTCACCA-5'), and a plasmid-specific primer, Tn7R109 (3'-CAGCATAACTGGACTGATTTCAG-5'), which give an amplicon of ~750 bp long. A typical 50 µl PCR mixture is listed in Table 2.2, with the dNTP mixture prepared from a dNTP set (Bioline) containing four separate 100 mM dNTP solutions.

PCRs were carried out using a gradient thermal Palm-CyclerTM (Cobett Life Science) according to the thermal cycle shown in Table 2.3.

Table 2.2: Reagent list for a typical 50 µl PCR mixture.

	Final concentration	Volume (µl)
10x PCR Running Buffer	1X	5
$MgCl_2$ (50 mM)	1.5 mM	1.5
dNTP (10 mM)	0.2 mM	1
Forward primer (10 µM)	0.2 µM	1
Reverse primer (10 µM)	0.2 µM	1
Taq DNA polymerase (5U/µl)	1U	0.2
Template DNA	1-5 ng	5
MilliQ H_2O	-	35.3
Total volume	-	50

Table 2.3: Thermal cycle for PCRs.

	Temperature (°C)	Time	Cycles
Initial denaturation	94	5 mins	1x
Denaturation	94	45 s	
Annealing	56	45 s	35x
Elongation	72	1 min/kb	
Final elongation	72	10 min	1x
Hold	4	-	-

Quantifying bacterial infectivity/virulence through bioreporters

A newly developed method using *lux*-labelled bacterial strains as a bioreporter to quantify *Psa* infectivity or virulence is proposed in this work. This biological tool can be used in different types of infection assay procedures and is described below.

Detached leaf assay

The P_{nptII}-*lux*-labelled *Psa* culture was prepared as follows: overnight culture was centrifuge at 4000 g for 10 mins and altered to A_{600}=0.2 by resuspended in nutrient-free 10 mM $MgSO_4$. The surfactant, Du-Wett, was added to the culture to a final percentage of 0.001% to help the spreading on leaf surfaces. If the leaf samples needed to be treated (e.g., with bactericide), Du-Wett was also added to the same final percentage.

Mature leaves from soil-grown plants were harvested and dip-inoculated in the prepared *lux*-labelled *Psa* suspension. Leaves were air-dried and sprayed with treatments on both sides (adaxial and abaxial surfaces) of the leaf (if needed) until the surfaces were wet but no runoffs. Inoculated leaves were then placed on square Petri dishes with wet filter paper surrounding the plate sides to maintain a humid interior environment. The dishes were incubated at room temperature for 2 days before capturing for bioluminescence signals. The procedure is outlined in Figure 3.2.

Flood inoculation assay

A bottle of 300 ml *lux*-labelled *Psa* culture was prepared as described in section 2.3.1. Young kiwifruit plants in agar pots were flood-inoculated with the prepared culture and swirled 5 times to ensure all leaves were fully inoculated with the culture. Culture in the agar pots was then drained and plants were air-dried. Inoculated young plants were incubated in a growth cabinet set to 22°C with a 14/10 day/night cycle for 10 days. All leaves from young plants were harvested and placed on a square plate for bioluminescence capturing by the CCD camera for *Psa* virulence quantification. This procedure is outlined in Figure 2.1.

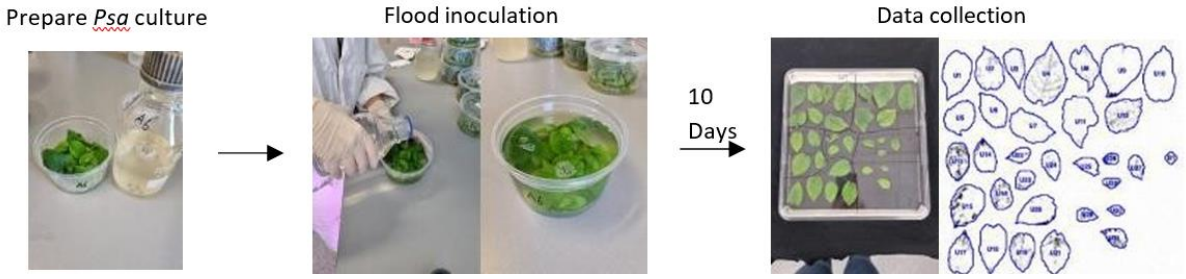


Figure 2.1: The procedure for flood inoculation assays. *lux*-labelled *Psa* culture was altered to A600=0.2 and supplemented with 0.001% of Du-wett to support the spread of culture on leaf surfaces. Prepared culture was then poured into the agar pots containing young kiwifruit plants and swirled 5 times to ensure even inoculation of leaves. Inoculated plants were incubated in a growth cabinet set to 22°C with a14/10 day/night cycle for 10 days. On the day of data collection, all leaves were harvested and placed ona square Petri dish to prepare for bioluminescence capturing using a CCD camera.

Quantification of bioluminescence

Bioluminescence signals from *lux*-labelled bacteria on leaf samples were quantified using a ChemiDoc Imaging System (Bio-rad). Leaf samples were placed inside the machine for dark treatment for 5 mins to remove plant autofluorescence. A charge-coupled device (CCD) camera was used for capturing bioluminescence signals with an exposure time of 200 s.

Resulting images were quantitively analyzed using the 'volume tool' of image Lab™ software (Bio-rad). The intensity of bioluminescence was calculated using the equation: the total signal from leaf sample/ exposure time/ area of the leaf sample.

Phenotypic characterization for evolved strains

Copper resistance determination

Assessing the Minimal inhibitory concentrations (MIC) of CuSO4 for P. fluorescence SBW25 evolved strains

Fresh LB media supplemented with various $CuSO_4$ concentrations (0, 0.1, 0.5, 1, 1.5,

2, 2.5, 3, 3.5, 4, 5, and 6 mM) were prepared. Overnight bacterial cultures were inoculated into these LB media in a ratio of 1:100 and mixed well. Bacterial suspensions were grown in a 96-well microtiter plate with 200 µl in each well and at least three technical replicates were included for each condition. The growth of bacteria was monitored using a Synergy^{TM} 2 multi-mode plate reader with an absorbance wavelength of 450 nm (A450) (because ionic copper interferes with light at wavelength 600 nm (A600)) at 28°C for 24 hrs. Cell density data were used for MIC calculation.

Assessing the MIC of CuSO4 for *Psa* evolved strains

Fresh LB media supplemented with varying concentrations of $CuSO_4$ (0, 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, and 6 mM) were prepared. Overnight bacterial cultures were inoculated into these media in a ratio of 1:100 ratio. Bacterial suspensions were grown in a 96-well microtiter plate with 200 µl per well and at least three technical replicates were included for each growing condition. The plate was incubated at 28°C for 14 hrs. and the bacterial density was detected at A450 using a Synergy^{TM} 2 multi-mode plate reader. The A450 data were then used for MIC calculation.

Antibiotic resistance determination

Epsilometer test (commercial strip) method

The *Epsilometer* test (Glupczynski et al., 1991) is commonly used for determining the MICs for antibiotics. A commercial antibiotic strip (Liofilchem), which contains a gradient concentration of an antibiotic, was used in this work to detect the MICs of ciprofloxacin, colistin, and tobramycin for evolved strains. An overnight culture was evenly streaked on an LB agar plate with a cotton swab three times. Between each streak, the plate was turned 60˚ with no re-dipping of the swab into the culture. Next, the antibiotic strip was placed in the center of the plate and the inoculated plate incubated at 28°C for 24 hrs. The MIC of antibiotic of the streaked strain can be determined by the intersection between the inhibitory zone and the lowest reading on the strip (Figure 2.2).

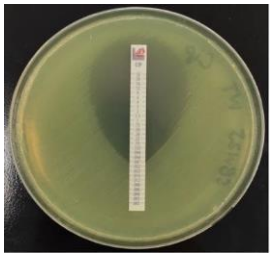


Figure 2.2: Example of P. fluorescens SBW25 growth on an LB agar plate containing a ciprofloxacin strip. A ciprofloxacin strip (Liofilchem) was placed in the middle of the plate after streaking with the SBW25 culture. The intersection between the inhibitory zone and the strip is the MIC of ciprofloxacin for this bacterial strain. Here, the MIC is 0.047 mg/ml.

In-broth method

Pre-experiments were done to determine a suitable range of concentration for each antibiotic. This was done by monitoring the growth of *P. fluorescens* SBW25 in LB supplemented with varying concentrations of antibiotic using a Synergy^{TM} 2 multi-mode plate reader at A600.

A total of six antibiotics were tested for SBW25 evolved strains in broth assays (Table 2.4). Laboratory media were prepared by mixing fresh LB with varying concentration of antibiotics. Overnight cultures of SBW25 evolved strains were inoculated into these media in a 1:100 ratio. Bacterial cultures were then transferred to 96-well microtiter plate with 200 µl per well and at least 4 technical replicates for each growing condition. Cultures were then incubated at 28°C for 9 hrs. before examining the cell density using a plate reader at A600. The A600 data were then fitted to the Gompertz-MIC model for MIC and MNIC calculation.

Table 2.4: List of antibiotics used in this study.

Antibiotic	Stock concentration (mg/ml)
Tobramycin	10
Gentamycin	20
Nalidixic acid	30
Kanamycin	50
Rifampicin	10
Streptomycin	10
Tetracycline	10

Calculation model of the MIC and MNIC for bactericide, $CuSO_4$, or antibiotics.

The Gompertz-MIC model, in conjunction with GraphPad Prism 9 software, was used to calculate the MIC and maximum non-inhibitory concentration (MNIC) of supplements for bacterial strains.

To determine the MIC of $CuSO_4$ or antibiotics in phenotypic assays, bacterial cell density data in the form of absorbance (A600 or A450) were collected from growth assays and fitted to the Gompertz-MIC model for calculation. Bioluminescence intensity (count/sec/area) was used to determine the MNIC of bactericide for *Psa* infection and growth *in planta*.

Genome re-sequencing and analysis

All copper-selected *Pseudomonas* strains were subjected to whole genome sequencing at Novogene (Beijing, China) with Illumina NovaSeq6000. Libraries were constructed from sheared DNA fragments of ~300 bp long and paired end reads at 150 bp were processed by Geneious 9.1.8 (Biomatters Ltd). Paired-end reads were trimmed using the BBDuk plugin in the software for further analysis.

Trimmed sequences were mapped to the reference genome NC_012660.1 (*P. fluorescence* SBW25), NZ_CP011972.2 (*Psa* NZ13), and NZ_CP0170091.1 (*Psa* NZ47) based on the evolved strains' ancestor using the "Medium-Low Sensitive/Fast" option in Geneious. Mutated genes were identified by "Fast Variations/SNPs" analysis with the following settings: "Minimum Variant Frequency" is 0.5, "Maximum Variant P-value" is 10^{-6}, "Minimum Strand-Bias P-value" is 10^{-5}, and "Find Polymorphism". All single nucleotide polymorphisms (SNPs) identified by the software were then checked with their variant frequency to ensure the value is higher than 0.97 before bioinformatic analysis.

Results

Development and validation of a method for quantifying bacterial infection of kiwifruit plants.

Copper-based bactericidal sprays are the most effective tool for controlling kiwifruit bacterial canker in orchards. However, with the emergence of copper resistant *Psa* strains, it is feared that the effectiveness of these sprays will be reduced. What is currently unknown is the role that copper bactericides play in suppressing *Psa* infection of kiwifruit plants. I hypothesize that plant infections by *Psa* can be suppressed by copper concentrations that have no effect on *Psa* growth and survival. Understanding the inhibitory effects of copper on *Psa* is crucial as the CuRrent use of copper bactericides is contributing to environmental pollution by heavy metals. A more comprehensive grasp of these mechanisms could pave the way for a more sustainable application of such bactericides.

To test the above-mentioned hypothesis, a new method based on *lux*-labelled bioreporters that allow the reliable quantification of plant infection by bacteria was developed. This method was used to determine the impact of bactericides on kiwifruit infection by *Psa* and the effect of these bactericides on the virulence of evolved Cu^R *Psa* strains.

Generation of Psa bioreporter strains.

To quantify plant infection by *Psa*, lux expression in planta was placed under the control of the constitutive promoter, P_{nptII}. The *nptII* gene, whose promotor exhibits constitutive gene expression in bacteria, codes for the enzyme neomycin phosphotransferase II that phosphorylates aminoglycoside antibiotics including kanamycin is a broadly use as resistance "marker".

All lab-evolved, *lux*-labelled bioreporter strains of *Psa* were generated by transformation according to the method described in section 2.2.1 and verified with PCR. In each case, the strain showed an expected PCR amplicon of ~750 bp in size after resolution by gel electrophoresis, indicating the *lux*-labelling was successful (Figure 3.1a and b).

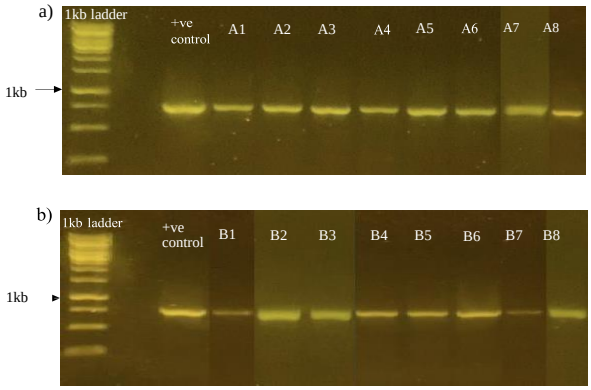


Figure 3.1: Polymerase chain reaction (PCR) verification of P_{nptII}-driven *lux*-labelled *Psa* reporter strains. The wild-type *lux*-labelled NZ47 strain (generated in a previous project) was used as a positive (+ve) control and 1kb ladder was used for determining the band sizes. a) NZ13-derived strains (A1-A8). b) NZ47-derived strains (B1-B8). Expected PCR amplicon size: 750 bp. The 1kb band on the ladder is labelled; the band below this is 750 bp in size.

Development of a method for quantifying bacterial infection of kiwifruit leaves.

A suitable method that can standardize the number of *Psa* cells during inoculation and the application of copper bactericides on the same kiwifruit leaf sample is required for reliable quantification of bacterial infection. Several methods were tested, including flood inoculation of young kiwifruit plants, leaf surface inoculation with a brush, and sponge stamping the leaf surface. For this study, I developed a detached leaf method to

examine the effects of copper-based bactericide application on *Psa* infectivity. The procedure is described in method 2.3.1 and Figure 3.2.

Briefly, kiwifruit leaves (Hort16A) were harvested from plants grown in soil and under well-controlled laboratory conditions: at 22 °C and 14/10 hours day and night cycles. These leaves were dip-inoculated in *lux*-labelled *Psa* culture (OD600=0.2) and resuspended in 10 mM nutrient-free $MgSO_4$ solution supplemented with 0.001% Du-Wett to help to spread the culture. Leaves were then air-dried at room temperature before being sprayed on both their adaxial (upper) and abaxial (lower) surfaces with copper until completely wet but not experiencing runoff. Bioluminescence signals were captured by CCD camera for analysis after 2 days.

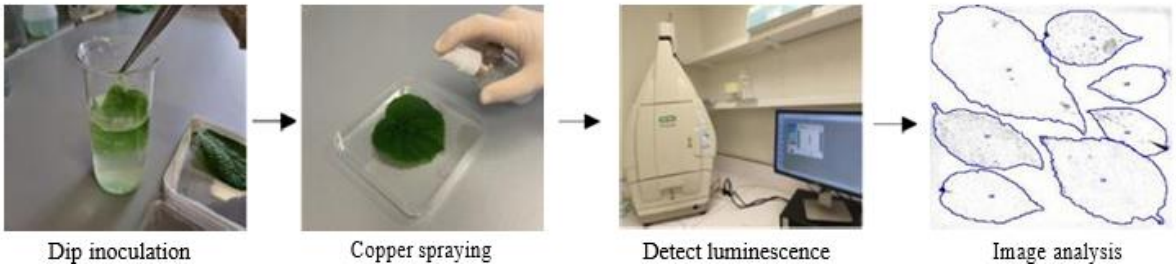


Figure 3.2: Outline of the detached-leave method used to examine the effects of copper-based bactericide application on *Psa* infectivity. Overnight culture of *lux*-labelled *Psa* was pelleted by centrifugation and altered to OD_{600}=0.2 and resuspended into 10 mM $MgSO_4$ supplemented with 0.001% of Du-Wett. Detached leaves from soil-grown kiwifruit plants were dip-inoculated into this culture and air dried at room temperature. Copper bactericides were sprayed on both sides of leaf until the surfaces were wet but not runoff. Bioluminescence signals were captured by a CCD camera.

Validation of the method used to quantify bacterial infection of kiwifruit leaves.

Along with the development of a new method for quantifying bacterial infection, several concerns were raised: **1)** whether the bioluminescence signal is from *lux*-labelled *Psa* or a signal from diseased leaf tissue independent form bacteria; **2)** whether the bioluminescence intensity is linearly proportional to the cell number of reporter bacterial strains; and **3)** whether this method can be used to comprehensively evaluate the degree of leaf infection by *Psa*.

I have performed a series of experimental assays to answer the above questions in this work as part of the new tool development.

Detected bioluminescence signal is from lux-labelled bacteria in planta.

To reliably quantify infection of kiwifruit leaves based on the *Psa*, bioluminescence signal captured by a CCD camera, this signal must arise from the lux-labelled *Psa* and not from

anything else such as the leaf itself or other bacteria that naturally from part of the leaf phyllosphere microbiota. Thus, an experiment was designed to compare the bioluminescence signal to the number colony-forming units (CFU) of non-pathogenic lux-labelled bacterium *Pseudomonas fluorescence* SBW25 inoculated into the leaf samples. This bacterial species was selected for this experiment as it is naturally unable to enter kiwifruit leaf tissue and, therefore, CFU number is more reliable as bacteria could be washed out easily. Inoculated leaf samples went through surface sterilization with the bactericide, Kocide (90 g/ 100 L), and then leaf disks were cut from treated leaves at different time-points after sterilization for bioluminescence determination with a CCD camera. At the same time-points, the P. fluorescens SBW25 on the leaf surface was collected by washing the leaf disks with 10mM $MgSO_4$ by vortexing.

The collected bacteria were titrated on agar plates to determine the number of CFU's collected from the leaf. Importantly, if bioluminescence is from *lux*-labelled *P. fluorescens* SBW25, the changing pattern of bioluminescence signal and CFU number in response to time should be the same (i.e., as the CFU number drops, so too should the level of bioluminescence).

Based on this experiment, I showed both bioluminescence signal and CFU number from the bactericide-treated kiwifruit leaf samples have reduced with the same pattern over time (Figure 3.3), indicating that the captured bioluminescence signal was from *lux*- labelled *P. fluorescens* SBW25.

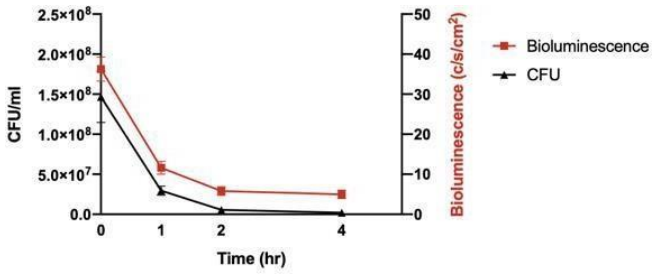


Figure 3.3: Comparison of the changing pattern intensity of bioluminescence signal and titer of CFU number on *lux*-labelled *P. fluorescens* SBW25 inoculated leaves of kiwifruit. The *lux*-labelled SBW25 was inoculated onto detached kiwifruit leaves and sterilized with Kocide (90 g/ 100 L). Leaf disks were cut from inoculated leaves at different time-points, *P. fluorescens* SBW25 was washed off from the leaf surfaces and CFU number was analyzed.

Bioluminescence signal is located at sites of *Psa* lesions on kiwifruit leaves.

The strongest evidence of kiwifruit leaf infection by *Psa* is development of diseases symptoms including leaf spots (lesions). To examine if the luminescence signal is derived from diseased leaf tissue infected with *lux*-labelled *Psa*, an experiment that observed and

compared the emergence of signal (by CCD camera) and bacterial lesions (by the naked eye) was carried out with the newly developed technique. I hypothesized that bioluminescence signal and *Psa* lesions will develop at the same position if signals are from diseased tissues.

Leaves from kiwifruit cultivar Hort16A were harvested and dip-inoculated with *lux*-labelled *Psa* NZ13 culture. The bioluminescence signal and *Psa* lesion data were collected daily for 10 consecutive days. The results showed *Psa* lesions developed at the same position on the leaf where the bioluminescence signal was captured after 2–4 days (Figure 3.4). In support of this, when a similar experiment was repeated on young kiwifruit plants grown in agar pots by another member in the group, the same results were observed (Appendix 1). The only difference here was that the *Psa* lesions were larger than those formed on detached leaves. This might be due to the difference in nature between detached leaves and whole living plants.

Together, the results of these experiments indicate that there is a direct linkage between bioluminescence signal and *Psa* lesion development on kiwifruit leaves.

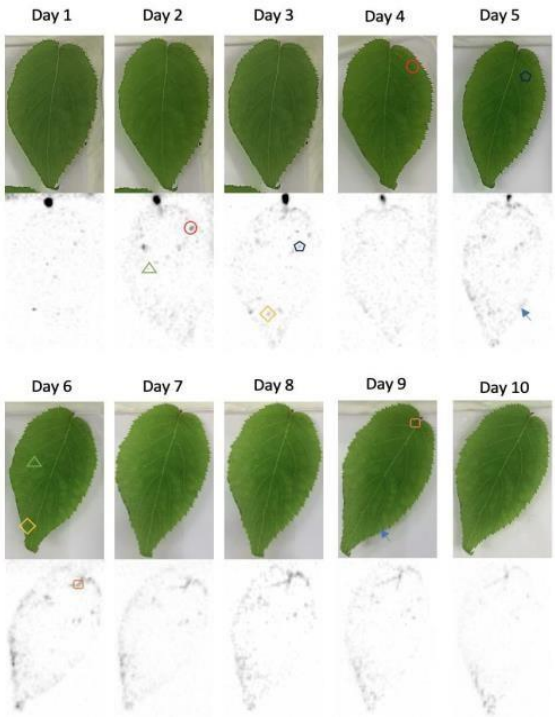


Figure 3.4: Location and development of bioluminescence signal and *Psa* lesion on kiwifruit leaves. Detached kiwifruit leaves from soil-grown cultivar Hort16A were dip-inoculated into lux-labelled *Psa* NZ13 culture and the development of bioluminescence signal together with *Psa* lesion were monitored by a CCD camera and by nude eyes, respectively, for 10 consecutive days. Shapes with the same coloring indicating *Psa* lesion and their corresponding bioluminescence signals.

Bioluminescence signal is from the interior tissues of infected kiwifruit leaves.

From the last section, I have successfully shown there is direct linkage between bioluminescence signal and *Psa* lesions on kiwifruit leaves. However, there is a concern that the bioluminescence signal is from colonization on inoculated leaf surfaces by the bacterium. Thus, I designed an experiment to compare bioluminescence signal before and after surface sterilization of the leaves. If the bioluminescence is partially from the colonization of the leaf surface, its signal would be expected to drop after surface sterilization.

The assay was conducted by dip-inoculating the detached kiwifruit leaves from soil-grown cultivar Hort16A into *lux*-labelled *Psa* NZ47 culture and then incubating these leaves at room temperature for 2 days. In doing so, *Psa* can infect the leaves and bioluminescence signal can be clearly captured by the CCD camera. Following incubation, the surfaces of the leaves were sterilized using a high concentration of copper bactericide, Kocide (90g/100L), which has been proven in a previous project to completely inhibit *Psa* NZ47 growth.

The results showed that the level of bioluminescence signal for each leaf was very similar before and after surface sterilization. This suggests that the bioluminescence signal originates from the leaf interior, where bactericide cannot reach, not from the leaf surface (Figure 3.5). This result further confirms that bioluminescence signal detected by the new kiwifruit leaf infection method is a good measure of infection by *Psa*.

Figure 3.5: Bioluminescence signal before and after surface sterilization of kiwifruit leaves inoculated with *Psa*. Detached kiwifruit leaves from soil-grown cultivar Hort16A were dip-inoculated into *lux*-labelled NZ47 *Psa* culture and incubated at room temperature for 2 days. Next, the leaves were surface sterilized through application of the bactericide Kocide (90g/100L) to both sides of the leaf. Bioluminescence signal was captured before and 30 minsafter sterilization by a CCD camera.

Bioluminescence signal is reduced in response to increasing copper concentrations.

To ensure the new technique would be able to quantify the concentration effects of copper bactericide on bacterial infectivity, a trial was done by treating *Psa*-inoculated leaf samples with bactericide at different concentrations. As bactericide can inhibit bacterial infection, the bioluminescence signal should show a negative relationship with bactericide concentration if this method is suitable for determining concentration effects.

Detached leaves from kiwifruit cultivar Hort16A were first inoculated with *lux*-labelled *Psa* NZ47 strain followed by Kocide treatment at varying concentrations. Bioluminescence signal was captured 2 days after inoculation. The results showed that with increasing copper concentration, bioluminescence signal is reduced (Figure 3.6), which supports this hypothesis and indicates that *Psa* infectivity in response to different copper concentrations can be determined with this method.

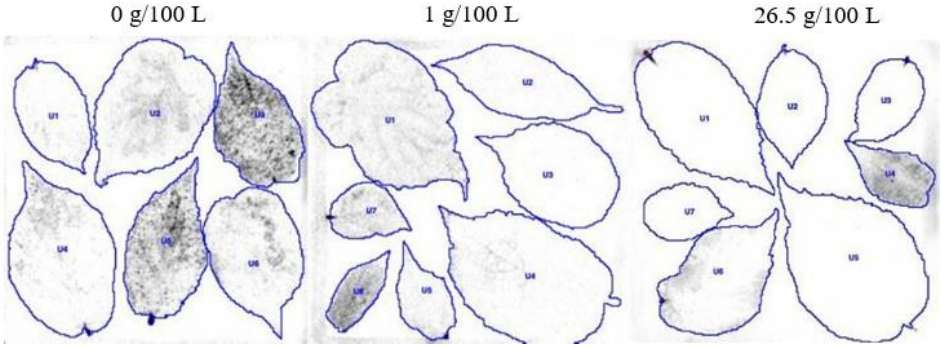


Figure 3.6: The change in bioluminescence signal observed from *lux*-labelled *Psa* infected kiwifruit leaves in response to different concentrations of a copper bactericide. The *lux*-labelled *Psa* strain NZ47 was dip-inoculated onto detached leaves of kiwifruit cultivar Hort16A, with the bactericide Kocide subsequently applied at concentrations 0, 1 and 26.5 g/100 L. Bioluminescence was captured using a CCD camera and compared 2 days after treatment with Kocide.

Bioluminescence signal is positively proportional to the diseased area of kiwifruit leaves infected with Psa.

The traditional method to examine the severity of bacterial infection on leaves is based on the ratio of diseased area to healthy area. Thus, it would be useful to show that bioluminescence signal is suitable for quantifying bacterial infection if the signal is indeed positively correlated to diseased area.

Young plants of kiwifruit cultivar Hayward, grown in agar pots, were flood-inoculated by *lux*-labelled *Psa* NZ13 culture. Data for bioluminescence and diseased area (%) were collected from all leaves (n=368) and analyzed by Image Lab or Image J software respectively. Results from this experiment showed a moderate positive correlation (r= 0.52, P-value= <0.0001) between bioluminescence signal and infected area (Figure 3.7), indicating the novel bioluminescence method is complementary to the traditional diseased area method. However, I have found that the two methods cannot substitute each other because bioluminescence signal corresponds to the early detection of infection, where the signal could be strong when no *Psa* lesions are observed, while signal drops when diseased tissue is dead.

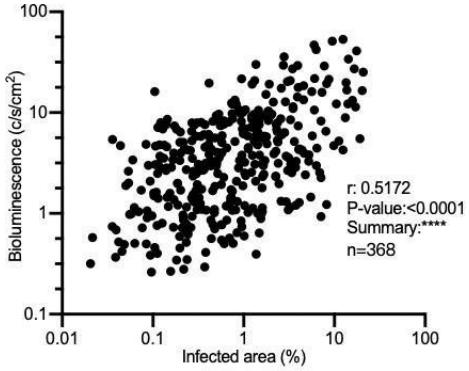


Figure 3.7: Correlation of bioluminescence signals and diseased area of kiwifruit leaves infected by *lux*-labelled *Psa*. Plants of agar-grown kiwifruit cultivar Hayward were flood-inoculated with *lux*-labelled *Psa* NZ13 culture. Bioluminescence and diseased area were determined by Imgae Lab and Image J software 10 days after inoculation, respectively. The correlation strength between the 2 data sets in moderately positive (statistical detail: n=368, r=0.52, P-value<0.0001).

Examination of the role of copper in inhibiting *Psa* infectivity.

The role of copper in inhibiting *Psa* infectivity in kiwifruit plants remains elusive. It is highly likely that copper bactericides suppress *Psa* infection at concentrations that have no effect on *Psa* growth (non-inhibitory concentrations). To test this hypothesis, the detached leaf method together with the *Psa* bioreporter were used for determining and comparing the maximum non-inhibitory concentrations (MNIC) for *Psa* infection and growth on kiwifruit leaves.

The experiment to establish the concentration effect of bactericide on *in planta* growth of *Psa* was demonstrated by Dr. Naren in a previous project. Leaves were harvested from plants of kiwifruit cultivar Hort16A or Hayward grown in soil and treated with varying concentrations of Kocide (0, 0.0009, 0.009, 0.09, 0.45, 0.9, 2.25, 4.5, 9, 18, 36, 54, 72, 90, and 135 g/100 L). Samples were cut from treated leaves as leaf disks and 5 µl of 10 mM-$MgSO_4$-suspended *lux*-labelled *Psa* NZ47 culture was dropped onto each disk. Bioluminescence signal was then captured after 2 h of incubation at room temperature.

On the other hand, the detached leaf method (Figure 3.2) was used for assessing the inhibitory effects of Kocide on *Psa* NZ47 infectivity. The *lux*-labelled *Psa* NZ47 culture was inoculated onto detached leaves of kiwifruit cultivars Hort16A and Hayward grown in soil and treated with a series of Kocide concentrations (0, 0.05, 0.1, 0.25, 1, 5, 16, 26.5, and 90 g/100 L). Bioluminescence signal was then captured 2 days after the treatment.

The maximum non-inhibitory concentration (MNIC) values for both *Psa* growth and infectivity *in planta* were calculated by fitting the bioluminescence data to the Gompertz model. For both cultivars, the Kocide MNIC values for *Psa* NZ47 infection (Hort16A: 0.09, Hayward: 0.13) was lower than that for *Psa* NZ47 growth (Hort16A: 4.96, Hayward: 6.25) (Table 3.1 and Figure 3.8). These results support the hypothesis that copper bactericides can inhibit *Psa* infection before growth being affected on kiwifruit leaves.

It is interesting that kiwifruit cultivars do make a difference in terms of bioluminescence reading. It is highly likely due to the physical physiology different between the cultivars. By observations, Hayward's leaves have more and stronger hairy structure on both sides of the leaves when comparing with the leaves of Hort16A plants. This difference could lead to higher bactericides loading capacity on the surface of leaves in Hayward resulting in greater effects on the growth of bacterial reporters causing a lower bioluminescence being detected in our results.

With this observation, it is important to keep all comparisons in later experiments within the same cultivars while investigating if chemical physiologies or other physical structures could involve in this observation could be another research area.

Table 3.1: The Kocide minimum inhibitory concentration (MIC) and maximum non-inhibitory concentration (MNIC) for *Psa* NZ47 infection and growth on leaves of kiwifruit cultivars Hort16A and Hayward.

Hort16A		
	Growth	**Infection**
MNIC (g/100 L)	4.96	0.09
MIC (g/100 L)	26.48	0.55

Hayward		
	Growth	**Infection**
MNIC (g/100 L)	6.25	0.13
MIC (g/100 L)	39.07	3.29

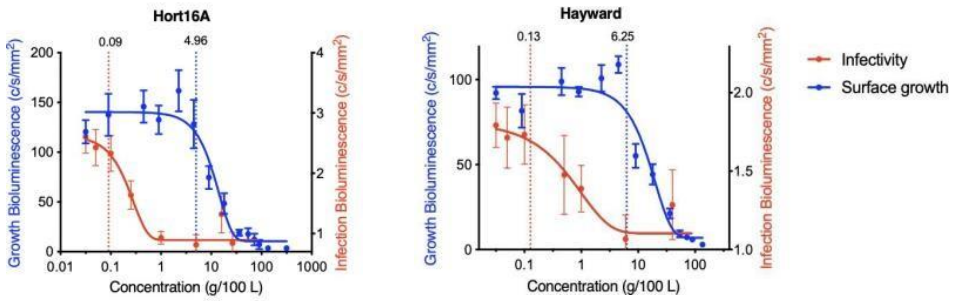


Figure 3.8: Kocide maximum non-inhibitory concentration (MNIC) fitting Curves for *Psa* NZ47growth and infection on leaves of kiwifruit cultivars Hort16A and Hayward. Detached leaves from soil-grown plants were harvested as leaf disks (growth assays) or whole leaves (infection assays). Samples were inoculated with lux-labelled *Psa* NZ47 and treated with various concentrations of Kocide. Bioluminescence signal was captured by a CCD camera after 2 h (growth) or 2 days (infection) of incubation at room temperature. The Gompertz model was used for fitting the bioluminescence data to calculate the MNIC values.

Experimental evolution of copper resistance in plant-associated *Pseudomonas*

To assess the potential of *Pseudomonas* in evolving copper resistance, the non-pathogenic, growth-promoting bacterium *P. fluorescens* SBW25 and the pathogenic bacteria *Psa* NZ13 and NZ47 were subjected to experimental evolution.

A unique method, colony-to-colony transfer, was adopted for these bacterial strains by growing them on linear-gradient plates. The gradient plates consisted of two angled layers: the bottom layer was plain LB agar, and the upper layer was LB agar with $CuSO_4$, which allows ionic copper diffusion from the upper to the lower layer, generating a surface with gradually changing Cu^+ concentration. A single colony growing on the sub-inhibitory area was selected for the next round of transfer (Figure 3.9). This experimental process was completed by an MSc student and Dr. Naren in previous projects, while the final evolved strains were chosen to complete phenotypic characterization in this work.

A total of five replicated lines with 30 transfers were derived from *P. fluorescens* SBW25 and eight parallel lines each with ~20 transfers were derived from *Psa* NZ13 and NZ47 strains.

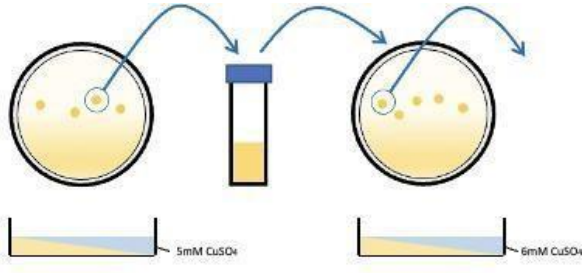


Figure 3.9: Outline of colony-to-colony transfer process. Bacterial culture was spread on the linear-gradient lysogeny broth (LB) agar plate supplemented with $CuSO_4$. A single colony that had grown on the sub-inhibitory area was selected and inoculated to LB for the next round of transfer with a slightincrease of concentration of $CuSO_4$ in the next agar plate.

Phenotypic characterization of the evolved strains after experiments evolution

Copper resistance

Resistance of evolved strains after experimental evolution to ionic copper was assessed by comparing the minimal inhibitory concentration (MIC) of copper ions between evolved strains and corresponding ancestral strains. The final generation of evolved strains were grown in LB supplemented with various concentrations of $CuSO_4$. Cell density at absorbance 450nm (A450) were fitted to the Gompertz model to calculate MIC values.

P. fluorescens SBW25

P. fluorescens SBW25 and its evolved strains were grown in LB with a series of $CuSO_4$ concentrations (0, 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, and 6 mM) in a 96-well microtiter plate. The growth of these strains was monitored using optical density at 450nm in a plate-reader (A450).

The MIC and MNIC values calculated by the Gompertz model using A450 data after 9 h of incubation are summarized in Table 3.2 (Appendix 2). All evolved strains had an increase of MICs by at least 18.7% and an increase of MNICs by at least 14.8% when compared to wild-type *P. fluorescens* SBW25, suggesting the evolved strains have increased Cu^R after colony-to-colony transfer. Furthermore, the evolved strains grew in 3.5 mM $CuSO_4$ supplemented LB when the ancestral wild-type strain was completely inhibited (Figure 3.10b) which is also indicating the evolved strains have better copper resistance.

Interestingly, I observed that the growth rate of evolved Cu^R strains in exponential phase was reduced in comparison with wild-type *P. fluorescens* SBW25 (Figure 3.10a). This might indicate that there is a trade-off between Cu^R and growth rate during evolution.

Table 3.2: $CuSO_4$ minimum inhibitory concentrations (MICs) and maximum non-inhibitory concentrations (MNICs) for *P. fluorescens* strain SBW25 and its copper-selected strains.

Values were predicted by fitting the A450 data from growing assays to the Gompertz model.

Strains	SBW25	AP	CP	DP	EP	FP
$CuSO_4$ MIC (mM)	2.978	4.112	4.763	3.822	4.472	3.645
$CuSO_4$ MNIC (mM)	1.863	2.755	2.204	2.472	2.775	2.187

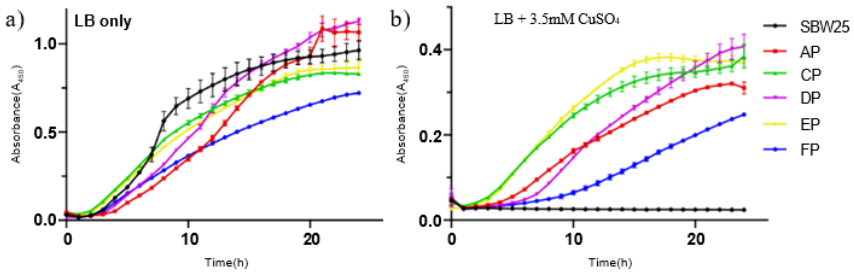


Figure 3.10: Growth Curves of evolved Cu^R and wild-type *P. fluorescens* SBW25 strains in lysogeny broth (LB). Five evolved Cu^R strains and the ancestral wild-type strain, SBW25, were grown without (graph a) and with (graph b) addition of $CuSO_4$. Bacterial growth was monitored by a plate-reader for 24 h at 28°C. Each points indicates the mean of four replicates and bars indicates the SEM.

Psa NZ13 and NZ47

After ~20 successful colony-to-colony transfers, the final generation of evolved *Psa* strains (eight replicates each from strains NZ13 and NZ47) were first assessed for copper resistance.

Each strain was grown in LB supplemented with varying concentration of $CuSO_4$ (0, 0.5, 1, 1.5, 1.75, 2, 2.25, 2.5, 3, 3.5, 4, and 5 mM) in 96-well microtiter plate without shaking for 14 h at 28°C. The A450 data was used for MIC and MNIC calculations. The results showed that the MIC values has increased by at least 26.4% and 30.6% in NZ13-derived and NZ47-derived strains, respectively, when compared to the corresponding ancestors (Table 3.3) (Appendix 3 and 4). This suggests the evolved strains have developed resistance to ionic copper.

Similar to the SBW25-derived strains, evolved *Psa* strains in general had a slightly slower growth rate in their exponential phase except A6, which had a large reduction in growth rate when compared with its ancestor (Figure 3.11). Again, this shows there may be a trade-off between Cu^R and bacterial multiplication.

Table 3.3: $CuSO_4$ minimum inhibitory concentrations (MICs) for *Psa* strains NZ13 and NZ47, as well as their evolved strains. Values were predicted by fitting the A450 data from growing assays to the Gompertz model.

Strains	NZ13	A1	A2	A3	A4	A5	A6	A7	A8
CuSO4 MIC (mM)	2.60	4.69	4.04	4.08	3.78	3.73	3.70	3.95	3.53
	NZ47	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**
CuSO4 MIC (mM)	2.99	4.85	4.50	4.40	4.43	4.32	4.38	4.91	4.67

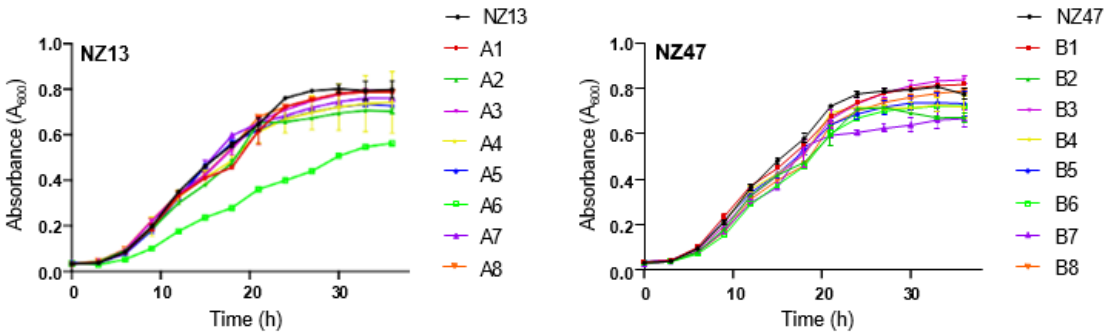


Figure 3.11: Growth Curves of evolved *Psa* strains and their ancestor in lysogeny broth (LB). Evolved strains and their ancestor were grown in LB for 38 h at 28°C. The A450 data was monitored by a plate-reader. Each point indicates the mean of four replicates and bars indicate SEM.

Antibiotic resistance

Many studies have shown that prolonged exposure of bacteria to high heavy-metal concentrations not only select for metal resistance but also select for antibiotic resistance. Thus, in this work, I assessed the potential correlation between copper and antibiotic resistance in *P. fluorescens* and *Psa* by comparing the MIC of different antibiotics between evolved Cu^R strains and their corresponding ancestors.

Two methods were used for this purpose: **1)** commercial antibiotic strips and **2)** in-broth assays.

Antibiotic strips

To determine the resistance of *P. fluorescens* and *Psa* to ciprofloxacin (Cip, fluoroquinolone family, 331.35 g/ mol), colistin (Cs, polymyxin family, 1,155 g/ mol), and tobramycin (Tob, aminoglycoside, 467.5 g/ mol), the MIC values for each evolved Cu^R strain was detected by Epsilometer test using ready-to-use antibiotic strips. Bacterial culture was evenly streaked onto LB agar plates by cotton swab until the surface was fully covered. The strip, which was placed in the center of the inoculated plate, was used for determining the MIC by reading the intersection between the strip and inhibitory zone after 24 h of incubation at 28°C.

P. fluorescens SBW25

The MICs of the three antibiotics for evolved *P. fluorescens* SBW25 strains are summarized in Table 3.4 (Appendix 5). These results suggest that all evolved strains have higher MIC values for Cip and Tob when compared to the ancestral SBW25 strain. This indicates that the evolved strains have developed better resistance to these two antibiotics.

However, resistance to Cs in copper-selected strains was highly variable. Four out of five evolved SBW25 strains (evolved strains were named as AP, CP, DP, EP and FP of which AP, CP, EP, and FP are the strains I am referring here) showed extremely high MIC values (>256 µg/mL) which exceeded the detection limit of the antibiotic strip. The DP strain, on the other hand, gave an MIC value that was half of the ancestral strain. In summary, the four strains have evolved to have higher Cs resistance, while the DP strain has evolved to have lower Cs resistance, indicating that most co-evolution between copper and Cs resistance is positive.

Table 3.4: Ciprofloxacin, Colistin, and Tobramycin minimum inhibitory concentrations (MICs) for copper-selected *P. fluorescens* SBW25 and its ancestral strain. The MICs were determined by the Epsilometer test using antibiotic strips purchased from Liofilchem.

	Strains					
Antibiotic MIC (µg/mL)	**SBW25**	**AP**	**CP**	**DP**	**EP**	**FP**
Ciprofloxacin	0.047	0.38	0.19	0.5	0.38	1
Colistin	12	>256	>256	6	>256	>256
Tobramycin	0.75	3	2	3	4	1

Psa NZ13 and NZ47

The MIC values for Cip, Cs, and Tob in evolved Cu^R *Psa* NZ13 and NZ47 strains are summarized in Table 3.5 (Appendix 6). These results show that all evolved strains have higher Tob MICs when compared with the ancestral NZ13 or NZ47 strain, indicating that evolved strains have developed greater Tob resistance after colony-to-colony transfer.

Similarly, all evolved *Psa* NZ13 strains exhibited increased MICs for Cs in comparison to its ancestor. However, only one evolved NZ47 strain (B3) had an elevated Cs MIC, while all the other strains showed reduced or consistent MIC values when compared to their

ancestor. This indicates that the copper-sensitive NZ13 strain has higher potential than the copper-resistant NZ47 strain for the co-evolution of copper and Cs resistances.

The resistance to Cip in both the NZ13- and NZ47-derived strains had no specific pattern. Three evolved *Psa* strains for each genomic background (NZ13-derived: A2, A6, and A7; NZ47-derived: B1, B3, and B8) exhibited increased MIC values, while other NZ13-derived strains, together with three NZ47-derived strains (B2, B5, and B6), showed the same Cip MICs as the ancestor. Furthermore, two NZ47-derived strains (B4 and B7) displayed a reduction in their Cip MIC. This suggests that the evolution of copper and Cip resistance occurs through different mechanisms.

Together, only four out of the sixteen evolved Cu^R *Psa* strains, specifically A2, A6, A7, and B3, exhibited increased resistance to all three antibiotics. These strains could be ideal models for studying the underlying mechanisms of co-evolution for copper and antibiotic resistance. To facilitate this, whole-genome re-sequencing should be performed.

Table 3.5: Ciprofloxacin, Colistin, and Tobramycin minimum inhibitory concentrations (MICs) for copper-selected *Psa* and its ancestral strain. The MICs were determined by the Epsilometer test using antibiotic strips purchased from Liofilchem.

	Strains								
Antibiotic MIC (μg/mL)	**NZ13**	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**
Ciprofloxacin	0.047	0.047	0.094	0.047	0.047	0.047	0.064	0.064	0.064
Colistin	0.25	0.5	1.5	1.5	0.5	0.75	0.5	0.75	1
Tobramycin	1	1.5	3	3	4	4	6	2	4

Antibiotic MIC (μg/mL)	**NZ47**	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**
Ciprofloxacin	0.047	0.064	0.047	0.064	0.032	0.047	0.047	0.023	0.064
Colistin	1	1	0.75	1.5	1	0.5	0.75	0.38	0.75
Tobramycin	1.5	3	3	4	3	4	6	6	6

In broth assays

The resistance of evolved *P. fluorescens* SBW25 strains to antibiotics under LB growth conditions was also determined. The A600 data after 9 h of incubation was chosen for MIC calculation.

Pseudomonas has natural resistance to ampicillin, carbenicillin, cephaloridine, cetrimide, and fucidin (Mohanty et al., 2021). Thus, these commonly used antibiotics were not included in these tests. A total of seven antibiotics, specifically tobramycin (Tob), gentamycin (Gm), nalidixic acid (Nal), kanamycin (Kan), rifampicin (Rif), streptomycin (Strep), and tetracycline (Tet), were tested in this work, with the results summarized in Table 3.6 (Appendix 7). All evolved Cu^R strains of *P. fluorescens* showed

increased MICs to Tob, Gm, Rif, Strep, and Tet when compared to the ancestral SBW25 strain. This result indicates that resistance to copper and these antibiotics likely co-evolves during the experimental evolution process.

On the other hand, four evolved strains of *P. fluorescens* exhibited a higher Nal MIC, while the AP-evolved strain demonstrated a slight reduction (from 15.57 to 13.44 µg/mL) in its Nal MIC, when compared with the ancestor. Similarly, the EP strain had a lower MIC value for Kan (from 2.44 to 1.60 µg/mL), while all the other evolved strains had raised MICs for Kan. Together, except for AP strain's resistance to Nal and EP strain's resistance to Kan, the resistance to all other tested antibiotics in evolved strains increased.

In addition to the absolute MIC values of antibiotics for each strain, the average fold change of antibiotics resistance in terms of MIC was also examined. In my study, all evolved strains showed increased MIC in five antibiotics (Strep, Tob, Gm, Rif, and Tet). Of these, the resistance to Strep, Gm and Tob in evolved strains have increased more than the other two antibiotics. Strep resistance of evolved strains exhibited the greatest change in MIC, with an increase of 13.2-fold. This was followed by Gm and Tob resistances with average increases of 7.29- and 2.77-fold, respectively. These three antibiotics are all aminoglycosides, while Rif and Tet belong to the rifamycin and tetracycline families, respectively. This indicates that resistance to members of the aminoglycoside family is more likely to co-evolves with copper resistance in *P. fluorescens* SBW25.

Table 3.6: Antibiotic minimum inhibitory concentrations (MICs) of copper-selected *P. fluorescens* SBW25 and the ancestral strains. The data were collected by examining A_{600} data after 9 h of incubation. The Gompertz-MIC model was used for calculation.

	Strains					
Antibiotic MIC (µg/mL)	**SBW25**	**AP**	**CP**	**DP**	**EP**	**FP**
Tobramycin	4.55	8.40	5.48	15.09	20.80	13.10
Gentamycin	3.86	5.25	15.78	41.13	41.12	35.20
Nalidixic acid	15.57	13.44	37.81	51.37	55.32	33.69
Kanamycin	2.44	3.81	3.00	5.47	1.60	4.48
Rifampicin	8.79	12.65	13.95	19.00	13.42	16.44
Streptomycin	5.36	>120	46.56	55.26	74.72	57.31
Tetracycline	2.49	6.46	3.86	4.57	6.44	10.74

Bacterial virulence

In section 3.1, I have demonstrated the use of *lux*-labelled *Psa* in infection or virulence assays is reliable. Here, I used these bioreporter strains to show the evolution of bacterial

virulence. To assess the potential correlation between copper resistance and bacterial virulence, an experiment was designed to compare the bioluminescence signals from leaf samples infected with *lux*-labelled *Psa*-evolved strains (described in section 3.1.1) and their corresponding ancestors.

Young kiwifruit plants of cultivars Hort16A and Hayward were, grown in agar pots, were flood-inoculated with evolved *Psa* NZ13 and NZ47 strains and their corresponding original ancestors, with all *Psa* strains being *lux*-labelled. All leaves from the seedlings were harvested for capturing of bioluminescence signal 10 days after inoculation. The intensity of the bioluminescence signal was then compared.

Bioluminescence signals from Hayward samples were lower than those from Hort16A samples when the same *Psa* strain was inoculated (Table 3.7). This result is expected as Hayward is a *Psa*-tolerant cultivar while Hort16A is a *Psa*-susceptible cultivar. Interestingly, bioluminescence intensity from samples inoculated with evolved strains of *Psa* was significantly lower than samples inoculated with the ancestor strains in both NZ13 and NZ47 (Figure 3.12). This indicates that there is less infection caused by evolved strains relative to the original ancestral strains or, in other words, the virulence of the evolved strains on kiwifruit has reduced. However, the degree of reduction varies and is cultivar-dependent (i.e., a strain that shows the greatest virulence reduction in Hayward could be a strain that shows only a moderate virulence reduction in Hort16A).

Table 3.7: The means of bioluminescence intensity (c/s/mm^2) from kiwifruit leaves infected with evolved *Psa* NZ13 and NZ47 strains and their corresponding ancestors.

				Strains					
Cultivar	**NZ13**	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**
Hayward	8.15	1.65	5.84	4.62	2.35	3.67	3.30	2.15	1.70
Hort16A	66.63	51.61	31.07	52.07	25.33	48.98	23.59	39.69	10.28
Cultivar	**NZ47**	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**
Hayward	12.85	8.68	0.74	0.81	1.29	5.05	2.07	1.26	7.69
Hort16A	37.56	25.82	25.12	7.35	29.52	26.27	29.54	25.99	23.73

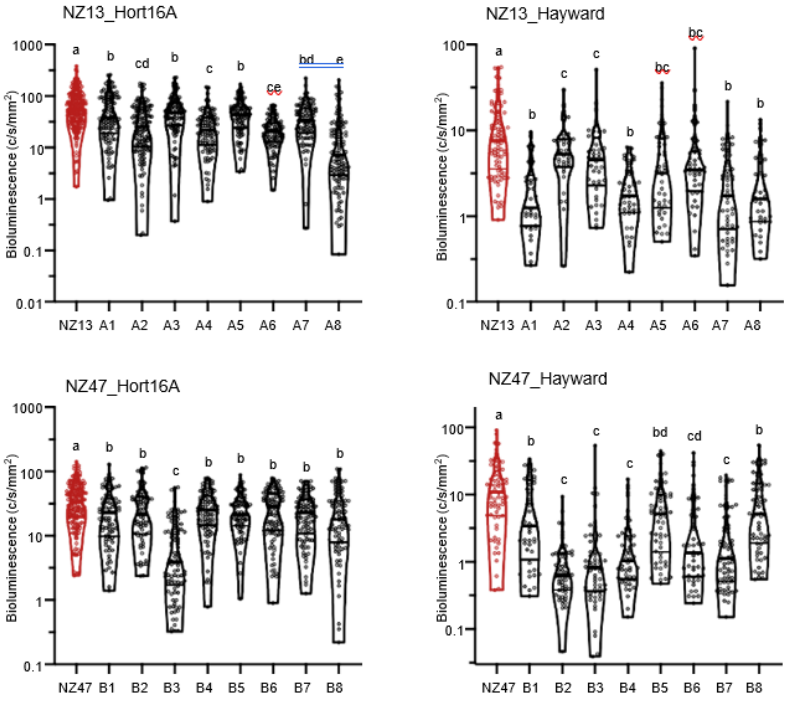


Figure 3.12: Violin plots illustrating the bioluminescence intensity from *Psa*-infected leaves of kiwifruit cultivars Hort16A and Hayward. Young agar plants were flood- inoculated with *lux*-labelled *Psa* (NZ13, NZ47, A1–A8, B1–B8) and signals were captured 10 days after inoculation with a CCD camera. A Tukey's multiple comparison test was used forstatistical analysis.

Genome re-sequencing

To investigate the underlying causes of the phenotypes described in section 3.3.1, all evolved *Pseudomonas* strains were subjected to whole genome sequencing using Illumina NovaSeq6000 technology (Beijing, China) and mapped by Dr. Naren to the reference genomes NC_012660.1 (*P. fluorescens* SBW25), NZ_CP011972.2 (*Psa* NZ13), or NZ_CP0170091.1 (*Psa* NZ47), depending on the ancestor. A set of single nucleotide polymorphisms (SNPs) with a frequency of >0.97 were identified.

Genome analysis for SBW25-derived strains of *P. fluorescens*

Five copper-selected SBW25 strains were subjected to genome re-sequencing and compared to the NC_012600.1 reference genome. I identified between two and eight mutations in each of the evolved strains. Variants of known gene products are listed in Table 3.8 and the complete set of identified variants is summarized in Appendix 8.

A total of 19 SNP-containing regions were identified across the five evolved strains. Among these, 16 were in coding sequences (CDS), with eight of these being well-studied genes (Table 3.8). In terms of the three mutations that ocCuRred in non-coding regions, one was in the promoter of the *infA* gene (PFLU3804), while the other two were

in intergenic regions (Appendix 8).

Interestingly, there were two gene loci, *copS* and 50S ribosomal protein L1 (*rplA*) gene show multiple sequence variations. CopS, a copper sensor kinase, had diverse mutations across the five evolved strains: more than one mutation was found in stains AP, CP and EP, with a single substitution of A to C ocCuRring at CDS position 830 in DP, resulting in a change of amino acid from leucine to arginine, and the addition of C at position 555 in FP causing a frame shift in the sequence. On the other hand, there were four variants of *rplA*, a ribosomal protein, in strains AP, CP, EP, and FP. In AP, multiple mutations were found, while different substitutions of nucleic acids were identified in other strains: an A to T at position 86 (resulting in change from leucine to glutamine) in CP, a C to T substitution at position 20 (change from arginine to histidine) in EP, and a C to T substitution at position 76 (change from alanine to threonine) in FP. CopS and RplA are known to play important roles in resistance to copper (Puig et al., 2002) and aminoglycosides (Calvopiña et al., 2019), respectively, in bacteria. This will be discussed in detailed in Chapter 4.

Furthermore, other genes that are well known to participate in antibiotic resistance such as *atpG* and *sdhA* were also found to be mutated in strains DP and FP, respectively. These genes might also contribute to the observed change in phenotypes of the corresponding strains.

Table 3.8: List of mutations in known gene products of evolved *P. fluorescens* SBW25 strains. Five SBW25-derived strains were subjected to genome re-sequencing and mapped to reference genome NC_012600.1. A set of mutations on known gene products were identified for each strain and are indicated by a tick symbol in the table.

		Strains				
Gene product	**Locus**	**AP**	**CP**	**DP**	**EP**	**FP**
CopS	PFLU1575	✓	✓	✓	✓	✓
RplA	PFLU5537	✓	✓		✓	✓
SpoT	PFLU5995	✓				
SdhA	PFLU1818			✓		
AraC	PFLU2563				✓	
LysR	PFLU2442				✓	
Htpx	PFLU1730				✓	
AtpG	PFLU6119					✓
Mean Coverage		161.7±25.9	159.6±26.2	163.7±26.9	156.2±25.7	157.4±26.5
Total no. of mutations		5	2	6	5	8

Genome analysis for Psa-derived strains

Genome sequencing of NZ13-derived Psa strains

The eight evolved CuR NZ13 *Psa* strains were subjected to genome re-sequencing and mapped to the reference genome NZ_CP011972.2 to identify possible mutations. These results showed there were between six and 11 mutations in each of the evolved strains. A full list of mutations is summarized in Appendix 9, while mutated genes with known functions are listed in Table 3.9.

A total of 30 mutated regions were identified across the eight evolved strains after mapping to the reference genome. Of these, 25 mutations (13 of which are relatively well studied, Table 3.9) ocCuRred in gene-coding regions, four in intergenic regions and one in the promoter region of the *hutC* gene (IYO_RS26430).

Three genes (*envZ*, *ompR* and *htpX*) showed multiple sequence variations across the evolved strains, while the other 10 mutated gene in Table 3.9 showed sole variation. EnvZ, an osmolarity sensor kinase, was mutated in four evolved strains (A2, A3, A4, and A5). In A2 strain the G at CDS position 440 was substituted by T causing the change of amino acid from serine to isoleucine. In A3 and A4 strains, a substitution of C to A ocCuRred at positions 1009 and 67, respectively. These mutations resulted in the change of amino acid from alanine to threonine and valine to methionine, respectively. In A5 strain, a T to C substitution at position 119 and a G to A at position 1078 ocCuRred, which led to the alteration of amino acid from valine to alanine and methionine, respectively. The response regulator of EnvZ, OmpR, showed two variants in A3 and A8 strains. Similarly, substitutions of nucleic acids ocCuRred in this gene. The substitution of C by T at CDS position 58 was identified in A3, while a G to T substitution at CDS position 268 was found in A8 strain. This caused an amino acid change from proline or alanine to serine, respectively. EnvZ-OmpR is a global regulator for osmolarity, copper and antibiotic resistance. Detailed will be discussed in Chapter 4.

On the other hand, HtpX (a zinc metalloproteinase that participates in aminoglycosides resistance) was mutated in strains A1 and A7. A substitution of C to A at position 402 ocCuRred in A1, leading to a change of amino acid from glutamic acid to aspartic acid, while there was a 6 bp deletion (GCGTTG) in strain A8. Although mutation on *hutC*

gene only found in A3, there is a mutation in the promoter region of the same gene in A6 strain, indicating HutC might have potential roles in copper resistance.

Table 3.9: List of mutations in known gene products of evolved *Psa* NZ13 strains. Eight NZ13-derived strains were subjected to genome re-sequencing and mapped to reference genome NZ_CP011972.2. A set of mutations in known gene products were identified for each strain and are indicated by tick symbol in the table.

		Strains							
Product	**Locus**	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**
EnvZ	IYO_RS01860		✓	✓	✓	✓			
OmpR	IYO_RS01855			✓					✓
HtpX	IYO_RS09125	✓						✓	
HutC	IYO_RS26430			✓					
SecD	IYO_RS06970	✓							
LysM	IYO_RS07750					✓			
OprD	IYO_RS28050							✓	
SulP	IYO_RS26860			✓					
PutA	IYO_RS25595			✓					
MpaB	IYO_RS23695			✓					
TetR	IYO_RS12405						✓		
Ribonuclease E	IYO_RS19850							✓	
Ribonuclease PH	IYO_RS00760								✓
Coverage means		152.7±46.3	150.2±31.8	164.9±52.7	161.9±57.5	153.2±37.1	157.9±42.9	154.4±33.8	155.2±41.4
Total no. of mutations		8	11	11	6	8	8	7	9

NZ47-derived strains genome sequencing

The eight copper-selected NZ47 strains were subjected to whole genome sequencing and mapped to the reference genome NZ_CP017009.1 to identify mutated genes. Nine locations exhibited substitutions across all evolved strains (Appendix 10). This raised the concern that the original ancestral strains already exhibited these mutations before the start of experimental evolution. This needs to be confirmed further by PCR or genome sequencing of the original strain.

Other than these nine regions, I identified 31 mutations across the eight evolved strains. Of these, 25 are within coding regions, while six are in intergenic regions. Three genes (*envZ*, *htpX*, and *kefA*) have multiple variants in the NZ47-derived strains, indicating that these genes could play crucial roles in copper resistance (Table 3.10). For *envZ* gene loci (PsaNZ47_RS02015), five out of eight evolved strains showed substitutional change in the nucleic acid sequence. Two of the strains, B2 and B4, have the same C to T substitution

at CDS position 89, which resulted in the change of amino acid from serine to phenylalanine. The B7 strain had two substitutions on the same gene, while B3 and B5 only harboured a single substitution of nucleotide on *envZ* gene. Interestingly, strains that did not have any mutation in *envZ* showed a change of DNA sequence in *htpX*. Both the B6 and B8 strains carried a deletion in *htpX* resulting a frame shift, while B3 was an exception, having mutation of both *envZ* and *htpX*. Furthermore, strains B2 and B4 had identical C to T silent substitution at position 345 in the cation-specific channel protein KefA.

Together, the data of all three genome sequencing assays have shown that the sensor kinase of two-component regulatory systems (CopS and EnvZ) could be the major target in the evolutionary process in response to ionic copper.

Table 3.10: List of mutations in known gene products of evolved *Psa* NZ47 strains. Eight NZ47-derived strains were subjected to genome re-sequencing and mapped to reference genome NZ_CP017009.1. A set of mutations in known gene products were identified for each strain and are indicated by tick symbol in the table. The common mismatching sections across all evolved strains are not counted in the total number of mutations.

		Strains							
Product	**Locus**	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**
EnvZ	PsaNZ47_RS02015		✓	✓	✓	✓		✓	
HtpX	PsaNZ47_RS09805	✓		✓			✓		✓
KefA	PsaNZ47_RS27535		✓		✓			✓	
HutC	PsaNZ47_RS28410					✓			
TerA	PsaNZ47_RS25620						✓		
LysM	PsaNZ47_RS08315						✓		
LysR	PsaNZ47_RS17110							✓	
DedA	PsaNZ47_RS28840			✓					✓
Coverage means		161.7± 58.3	155.3± 30.4	148.0± 49.1	150.5± 30.0	157.4± 44.6	160.7± 46.0	163.4± 41.8	159.2± 52.8
Total no. of mutations		2	4	4	9	5	5	8	4

Discussion

Copper is an essential trace element, but excessive copper can be lethal. The anti-microbial properties of copper have driven the development of copper-based bactericides, which are commonly applied to control a range of plant pathogens, including *P. syringae* pv. *actinidiae* (*Psa*) in kiwifruit orchards. However, the time and amount of copper required to reduce *Psa* infection *in planta* is not well-understood. In

addition, the extensive use of copper bactericides has been reported as promoting the emergence of copper-resistant strains. Thus, a worst-case scenario is that the continued and ever-increasing use of copper sprays in orchards would select for copper-resistant pathogens and thus, we will lack efficient control strategies for plant diseases moving forward.

In this work, a novel method using bioluminescence-reporter strains was developed to examine the amount of copper needed to inhibit *Psa* infectivity *in planta*. At the same time, a set of lab-evolved copper-resistant *Pseudomonas* strains were subjected to a series of phenotypic and genotypic assays to determine their evolutionary potential for copper resistance and the interplay with antibiotic resistance and virulence.

The validation of a novel bioreporter method to quantify pathogen infection.

In this work, a set of *lux*-labelled bioreporter strains were generated based on lab-evolved copper-resistant *Psa* strains and their wildtype ancestors *Psa* NZ13 and NZ47. These strains were used to determine the maximum non-inhibitory concentrations (MNIC) of copper bactericide on kiwifruit leaf infection by *Psa* and the virulence of the lab-evolved *Psa* strains. Thus, two methods, a detached leaf assay and a flood-inoculation assay for copper MNIC assays and *Psa* virulence assays, were developed.

Both methods were validated to ensure that the quantification of *Psa* infection *in planta* was reliable. I first showed that the bioluminescent signals captured on kiwifruit leaves by the CCD camera were exclusively from *Psa* cells. This specificity provided the foundation for me to calculate copper MNIC for *Psa* infection. Importantly, a direct linkage between bioluminescence and bacterial spots could be made, indicating that quantitative data on *Psa* infection could be collected. Taken together, *lux*-labelled *Psa* strains were found to be suitable for quantifying *Psa* infectivity.

This *lux*-based technique can also potentially be applied to early disease diagnosis as, in our pathosystem, bioluminescent signals emerged 2-4 days earlier than disease symptoms could be observed. Compared to traditional methods of quantification, such as polymerase chain reaction (qPCR) and immunofluorescence (IF), or visual inspection (Fang & Ramasamy, 2015), the novel methodology I developed in this work allows continuous, easy, and direct monitoring of crop diseases in early bacterial infection. This technique

also provides a platform for researchers to investigate influences during bacterial infection *in planta*.

However, like other biosensor tools, the strength of the bioluminescent signal in this technique relies strongly on the metabolic activities of the *lux*-labelled strains. This means severe plant tissue infections that reach the stage of decay would not be reliably detected. This is because the optimum conditions for bacterial activities no longer exist when tissues decay. In fact, this explains why samples could have low bioluminescent intensities when they have relatively large, infected areas (Figure 3.7). Unfortunately, when or at what stage the signals are no longer reliable has not been established yet.

In summary, the novel technique provides potential opportunities for early disease diagnosis and quantification of early- to mid-stages of bacterial infection *in planta*, but further studies are needed to identify the cut offs for reliable detection during mid- to late-infection stages.

Copper inhibits *Psa* infection at non-lethal concentrations.

The continued identification of copper-resistant pathogens in copper-polluted farmlands (Colombi et al., 2017) has raised a concern on the potential loss of effective practices to control crop diseases. This has, in turn, resulted in an ongoing evaluation of the use of copper for disease control. By determining the copper concentrations that effectively protect plants from bacterial infection, it may be possible to use copper in a more sustainable way without increasing the risk of copper resistance.

In contrast to other studies that have investigated copper efficiency by inferring bacterial survival rate in culture, I determined the MNIC for *Psa* growth and infectivity on kiwifruit leaves of two cultivars (Hort16A and Hayward) in response to Kocide (a copper-based bactericide commonly used in kiwifruit orchards). My results suggest that copper can effectively inhibit *Psa* infection at concentrations lower than those affecting survival (growth) *in planta*, indicating that the lethal concentrations of copper traditionally applied in farmlands are well more than what is needed to control bacterial infection. In doing so, my results might give a guideline to redesign the application level of copper bactericides in agriculture.

This set of results also raises fundamental research questions: what are the underlying mechanistic responses of bacteria to sub-lethal concentration of copper?

It is common for host plants to accumulate copper in their tissues as an innate defense against pathogens (Prohaska & Lukasewycz, 1981). Likewise, bacterial pathogens have evolved copper resistance systems to maintain copper homeostasis to increase their chances of survival during infection (Rowland & Niederweis, 2012). It is sensible that bacteria reduce their infectivity when exposed to sub-lethal concentrations of copper, as successful infection requires normal metabolism and functional enzymes. Indeed, enzymes such as dehydratases, which are responsible for branching amino acids chains, are the primary targets for copper during *E. coli* infection (Macomber & Imlay, 2009). Excess copper in the cell can displaces iron from iron-sulphur clusters of dehydratases and inhibits protein synthesis, leading to a pause in the infection process (Macomber & Imlay, 2009). Similar mechanisms might also apply to *Psa* during infection of kiwifruit, since the data here provide strong evidence that *Psa* infection was suppressed at low copper concentrations that do not affect growth. This indicates that some infection systems of *Psa* are disrupted by copper at sub-lethal copper concentrations.

Overall, a more sustainable practice of using copper bactericides in kiwifruit orchards could be developed with a practical view to reduce copper pollution and ease the problem of copper- resistant strains emerging. However, further studies focusing on investigating changes in physiology and gene expression via RT-PCR or transcriptomics are needed to answer the more fundamental questions.

The evolutionary potential of copper resistance in pseudomonads.

As mentioned in previous sections, one of the main concerns of using copper bactericides is the selection of copper-resistant strains (Petriccione et al., 2017). Nonetheless, the evolutionary potential, or maximum level, of copper resistance in bacteria has rarely been studied. In this work, a set of lab-evolved *Pseudomonas* strains (evolved from *P. fluorescens* SBW25, *Psa* NZ13, or *Psa* NZ47 via colony-to-colony transfer) were found to have evolved different levels of copper resistance.

After 15-20 transfers of the *Psa* strains, they appeared to be considerably close to the limit of their evolutionary potential for copper resistance. It was difficult to isolate single colonies from sub-lethal concentrations of copper during colony-to-colony transfer after ~20 transfers and, thus, the *Psa* strains appeared to have reached their evolutionary potential for copper resistance, which increased by between 30% and 60% compared to their wild-type ancestors. However, the evolutionary potential of copper resistance for *P. fluorescens* SBW25 could not be concluded at this stage. Due to the time limitations for this project, the experimental evolution work for SBW25 strains had to be stopped after 30 successful transfers, at which point single colonies were still easily isolated. After these transfers, copper resistance increased by ~20% in SBW25 evolved strains when compared to their ancestor.

Overall, despite more transfers, the evolved SBW25 strains demonstrated a lower level of copper resistance compared to evolved *Psa* strains. This might indicate that the evolutionary potential for copper resistance is lower in non-pathogenic bacteria than in pathogenic bacteria. A possible explanation for this phenomenon is the natural needs of pathogens to adapt to their host defense and immune system. Hosts can control the level of copper in infected areas of their tissue as a defense response, while pathogens need a strong copper resistance system to enable them to survive for possible infection (Liu et al., 2020). Beneficial bacteria on the other hand, such as *P. fluorescens* SBW25, have no survival needs to against hosts' defensive responses although they still required copper resistance to fight against high environmental copper. Thus, pathogenic bacteria might have higher evolutionary potential for copper resistance.

Up to this stage, I can conclude that copper resistance in *Psa* can increase by 30% to 60%. Continued experimental evolution will be needed to investigate the evolutionary potential of copper resistance in the non-pathogenic *P. fluorescens* SBW25. In addition, whole genome resequencing at different timepoints could help us understand the genetic changes and give insight into the differences in evolutionary processes between pathogenic and non-pathogenic bacteria.

Copper-induced mutations found in lab-evolved pseudomonads.

All lab-evolved copper-resistant strains were found to have an increased level of copper resistance. Such an increase can be connected to specific genetic changes which were investigated by whole genome resequencing. A set of mutated genes was identified for each of the evolved pseudomonad strains. More specifically, between two and eight mutations were found in evolved SBW25 strains, while two to 11 mutations were found in evolved *Psa* strains. Some of these mutations appeared in multiple strains, indicating these specific mutations and the regulatory or open reading frames (OFRs) they affected might have crucial roles in copper resistance.

Mutations in the *copS* gene were found in all *P. fluorescens* SBW25 evolved strains. This gene is a well-known sensory kinase that responds to copper and activates the expression of copper- resistance genes *copABCD* (Puig et al., 2002). The identified variants of *copS* might have enhanced the expression of these downstream genes and therefore resulted in the elevated copper resistance, although this hypothesis needs to be confirmed with site-directed mutagenesis and phenotypic assays.

Similarly, mutations in the *envZ* and *ompR* genes, the two-component regulatory system responsible for osmolarity and antimicrobial resistance (Ko & Choi, 2022), were found across most of the *Psa* evolved strains. This suggests that the EnvZ-OmpR regulatory system plays an important role in mediating copper resistance in *Psa*. However, there is no obvious direct link suggesting how this system is involved. It is possible that these ORFs act as global regulators that control the expression of certain copper resistance genes. Further studies such as site-directed mutagenesis, phenotypic characterizations of mutants, and transcriptomic assays in response to copper will be needed to unravel the linkage between EnvZ-OmpR and copper resistance mechanisms.

On the other hand, mutations at the ORF and regulatory region of the transcription factor, *hutC*, which is known to play a significant role in stress tolerance (Zhao et al., 2023), were identified in evolved *Psa* strains. It is possible that HutC participates in controlling the expression of stress-response factors, such as extracellular polysaccharide (EPS), when exposed to copper-induced stress (Zhao et al., 2023).

Together, a set of genes and their regulatory regions with well-established roles in copper resistance in pseudomonads, as well as others with unknown roles, was identified. Further investigations to conclude their importance and functions in response to copper exposure via site-directed mutagenesis, phenotypic assays and transcriptomic assays are needed.

Trade-offs between copper resistance and growth rate during evolution.

Copper overexposure is toxic for living cells, and this property is used for pathogen control in the agricultural sector. The emergence of copper-resistant strains is frequently documented in fields that have applied copper-based bactericides, but a possible relationship between copper resistance and other physiological characteristics, including growth rate, is rarely studied.

In this work, the growth rate of evolved copper-resistant pseudomonad strains was compared with their corresponding ancestors. The results showed that all evolved strains had a reduced growth rate in log and exponential phases, suggesting that there is a trade-off between copper resistance and growth rate. This might be explained by the level of energy and resources needed by the pseudomonad strains to effectively operate the copper-resistance systems.

Bacteria that evolved higher copper resistance likely have reduced copper influx, enhanced copper efflux, and/or undergo copper sequestration (Cervantes & Gutierrez-Corona, 1994). However, the increased ability of efflux and sequestration systems means extra energy and resources are required to maintain the functions of relevant proteins which would reduce those for normal bacterial growth (Irawati et al., 2021). In fact, similar observations have also been reported in *Acinetobacter* sp. IrC2 (Irawati et al., 2021).

In summary, copper resistance mechanisms allow bacteria to survive in copper-rich environments, but these mechanisms can slow down their growth rate due to resources depletion. This trade-off has important implications for understanding bacterial evolution and thus the identification of genes that might be involved in growth regulation is important. These genes might give some clues on understanding bacterial strategies

associated with trade-offs between resistance and growth.

The co-evolution of copper and antibiotic resistances in pseudomonads.

Many copper-resistant bacterial strains have also been reported to have higher antibiotic- resistance compared to their wild-type ancestors (Baker-Austin et al., 2006). Similar results were observed in this work here. The results of whole genome resequencing might give some explanations to this observation.

Almost all five lab-evolved *P. fluorescens* SBW25 copper-resistant strains have increased resistance to ciprofloxacin, colistin and tobramycin compared to their ancestors, while only four lab-evolved *Psa* strains had higher resistance compared to their ancestors in the antibiotic strip test. This might indicate that the co-evolution of copper and antibiotic resistance in pathogenic bacteria is weaker than in non-pathogenic bacteria, although it is generally believed that pathogenic bacteria often evolve with antibiotic resistance more rapidly (Merker et al., 2020). However, since evolutionary mechanisms are complex, and no experiments were designed to directly compare the co-evolution of copper and antibiotic resistance in pathogenic and non-pathogenic bacteria, there is no strong evidence yet for this hypothesis.

Another six antibiotics: gentamycin, nalidixic acid, kanamycin, rifampicin, streptomycin, and tetracycline (tobramycin was also included) were tested on the lab-evolved *P. fluorescens* SBW25 strains in broth media. Resistance to tobramycin, gentamycin, and streptomycin increased the most across all lab-evolved *P. fluorescens* SBW25 strains (Table 3.6). These three antibiotics are all aminoglycosides, suggesting resistance to copper and aminoglycosides co-evolved spontaneously in *P. fluorescens* SBW25. Indeed, research has shown that there is co-ocCuRrence of resistance genes that belong to copper- and aminoglycoside-resistance systems in bacteria (Pal et al., 2015), and that aminoglycosides can target host RNA motifs with the combination of copper to cause irreversible degradation of the RNA (Patwardhan & Cowan, 2011). Genomic data from evolved strains could add supportive evidence to this hypothesis.

Genome re-sequencing data of the lab-evolved *P. fluorescens* SBW25 strains identified

mutations in *copS* and *rplA* across all evolved strains. These encode a copper-responsive sensor kinase and a ribosomal protein, respectively. This indicates that the two genes potentially have important roles in both copper and aminoglycoside resistance in bacteria. In fact, RplA is known to play a crucial role in the production of the aminoglycoside efflux pump, SmeYZ, in *S. maltophilia* (Calvopiña et al., 2019). Furthermore, a proteomic study in *E. coli* showed that exposure to copper dramatically reduced expression of the RplA protein (Nandakumar et al., 2011), indicating it is highly likely to have a direct linkage between copper- and aminoglycosides-resistance at the molecular level, although the precise mechanistic linkage has not yet been discovered. It is possible that decreased expression of this ribosomal protein could lower the overall metabolic rate of bacteria and therefore prolong life span in high copper concentration environments. The *copS* gene encodes the copper-induced sensor kinase of the CopRS two-component regulatory system that is responsible for copper resistance in pseudomonads (Puig et al., 2002). Two- component regulatory systems are commonly found in bacteria for precise and rapid responses to environmental stress. Research has found that a two-component regulatory system in *A. baumanii*, AdeRS, is responsible for both osmolarity control (Fernando & Kumar, 2012) and aminoglycoside resistance (Ruzin et al., 2007), indicating that two- component regulatory systems acting as global regulators to govern different cellular systems is common in bacteria. Thus, the potential of the CopRS system having cross-over roles in copper- and aminoglycoside-resistance cannot be overlooked. Further studies on transcriptomic and proteomic profiles of *copRS* mutant strains might give some clues on this topic.

A different set of mutations in genes of known function were identified in lab-evolved *Psa* strains. Among these were *envZ* and *ompR*, encoding for a two-component regulatory system, which harbored mutations in 10 out of 16 of the evolved *Psa* strains. This high evolutionary parallelism, together with the general increased resistance to ciprofloxacin, colistin, and tobramycin, suggest that a possible mechanistic connection between copper- and antibiotic- resistance might ocCuR through mutations in the EnvZ-OmpR system. As a matter of fact, this system is responsible for controlling the expression of porins (products of *ompC* and *ompF* genes), recent research has proven both direct and indirect participation in enhancing antibiotic resistance by remodeling outer membranes

protein composition to reduce membrane permeability in *S. enterica* (Ko & Choi, 2022). Furthermore, mutations in *envZ* were found to compromise the binding and entry of the antimicrobial peptide, TAT-RasGAP317-326, leading to an increase of antimicrobial resistance in the model organism *E. coli* (Georgieva et al., 2022). Together, it is likely that *envZ* and *ompR* could be global regulators governing the expression of copper- and antibiotic-resistance genes in *Psa*.

Up to this stage, my work has provided multiple lines of evidence that the evolution of copper- and antibiotic-resistance are linked in pseudomonads. This could be via two-component regulatory systems such as CopRS and EnvZ-OmpR, which are commonly found in bacteria and have been proven to have multiple functions in maintaining cell metabolism. However, to understand the dual roles of these proteins in copper- and antibiotic-resistance, more research such as protein interaction assays and transcriptomics will be needed.

The reduction of virulence in lab-evolved copper-resistant *Psa* strains.

Both animal and plant hosts can increase intracellular and extracellular levels of copper to protect themselves from pathogenic infection (Chaturvedi & Henderson, 2014). Bacteria, on the other hand, have evolved copper resistance mechanisms such as efflux pumps and sequestration proteins to guard against the hosts' defense system (Chaturvedi & Henderson, 2014). From this, I hypothesized that the more resistance the pathogen has against copper toxicity, the better it can infect its host.

Interestingly, my findings go against my original hypothesis. I have shown that the evolved copper-resistant *Psa* strains have reduced virulence compared to their wild-type ancestors (section 3.3.1.3), indicating that the ability to infect host plants decreased in the copper- resistant strains. There are two possible explanations for this observation.

Firstly, energy and resource depletion might be a cause. To maintain a high copper-resistance level in bacteria, energy and resources might be drained from other metabolic systems including those associated with the infection process (Irawati et al., 2021). This leads to a trade- off between copper resistance and virulence in the lab-evolved *Psa* strains. Secondly, my experimental design might not totally fit my purpose. I have

shown that there is a trade-off between copper resistance and growth rate in the previous section, but the growth rate difference between lab-evolved and wild type control strains wasn't considered in virulence assays. It is possible that the reduced growth and multiplication rate of lab-evolved strains delayed the infection process. Thus, the infection impacts of evolved strains appeared less severe when compared with their wild-type ancestors at the same given time-point.

However, my genome re-sequencing data might explain this phenomenon to some degree. Several transcriptional regulators, including *envZ*, *ompR*, *hutC*, *secD* and *kefA*, which are known to have significant roles in regulating the expression of virulence factors (Pasqua et al., 2022; Quiblier et al., 2011; Zhu et al., 2021), were found to be mutated in the genomes of the lab-evolved *Psa* strains. A mutation in a more directly related gene, *lysM* which encodes a virulence factor that suppress chitin-triggered immunity in plants during infection (Cen et al., 2017), was also found in the lab-evolved *Psa* strains. Although this set of genes could provide an explanation regarding the reduced virulence in evolved strains, exactly how these mutations change the functions of the proteins they encode and the evolutionary potential of virulence in *Psa* remains unclear.

Conclusion

This work aimed to improve our understanding of the molecular and evolutionary mechanisms of copper resistance in plant-associated pseudomonads. Through development of *lux*-labelled *Psa* strains, I was able to demonstrate that inhibition of *Psa* infection by copper-based bactericides comes before lethal concentrations are reached. Importantly, this knowledge contributes to the sustainable practice of copper-based bactericide application in kiwifruit orchards, as my results suggest that lower levels of bactericide than CuRrently used are sufficient to control *Psa* infection.

On the other hand, my work has shown that long-term exposure to copper (with evolution via colony-to-colony transfer) can promote the emergence of copper resistant pseudomonad strains. In addition, co-evolution of copper- and aminoglycoside-resistance was also illustrated. However, the full evolutionary potential of copper resistance in *P. fluorescens* SBW25 has not yet been demonstrated. Through the identification of

mutations, set of genes that encode two-component regulatory systems (CopRS and EnvZ-OmpR) and transcription factors (HutC and KefA), all of which have known functions in stress tolerance, antimicrobial resistance, and virulence, were identified that help to explain the phenomena. These results provide new insights into a potential relationship between copper resistance, antibiotic resistance, and pathogenic virulence at the molecular level.

Future potentials and works

I have identified genes and regulatory participants involving in copper resistance via studying the copper resistance mutants in this work. It is interesting that most of the mutations were not belonging to the known copper resistance systems indicating a huge knowledge gap is lying within this area. To close the knowledge gap, further research focusing on mutagenesis with phenotypic characteristics, transcriptomic analysis and protein interaction assays is needed to specify each gene's function in response to copper exposure.

On the other hand, previous studies have shown the potential relationship between heavy metals resistance and antibiotic resistance. I have identified the positive relationship between copper resistance and aminoglycosides resistance in *P. fluorescence* SBW25 via phenotypic assays in this work which has narrowing down the direction. A set of potential candidate genes, which some of them are global regulators, that might be involved in this positive relationship was identified via genome re-sequencing in this project. Further investigations on how these possible regulators participate in the genetic regulation processes via mutagenesis and protein interaction assays could unravel the potential underlying dual-evolution pathways of copper and aminoglycosides resistance.

It is also necessary to study the antibiotic resistance for both bacterial strains *in planta*. Copper and antibiotics are both commonly used antimicrobial substances in agriculture. However, the efficacy of these disease prevention practices *in planta* is unclear as bioavailable copper or antibiotics could be different when applied to media culture and plant surfaces. In this work, the efficacy of copper-based bactericides on *Psa* in kiwifruit leaves were investigated with the newly developed bioreporter tool. This tool could be also

used for detecting other antimicrobial substances on plants after moderation is made.

Finally, I have observed the decrease in virulence on kiwifruit leaves of pathogenic copper-resistant *Psa* strains. It is highly likely due to the slower growth of copper-resistant strains which has been proven in broth culture. I have hypothesis this is due to the reduce of fitness in situations without copper which could be a trade-off during copper-resistant evolution. Further observations and analysis are needed via planned fitness tests to make a conclusion.

Overall, Pseudomonad responds towards copper and antibiotics requires further researching focusing on phenotypic characterization of mutants and transcriptomic assays to improve our understanding of copper-based antimicrobials used in agriculture.

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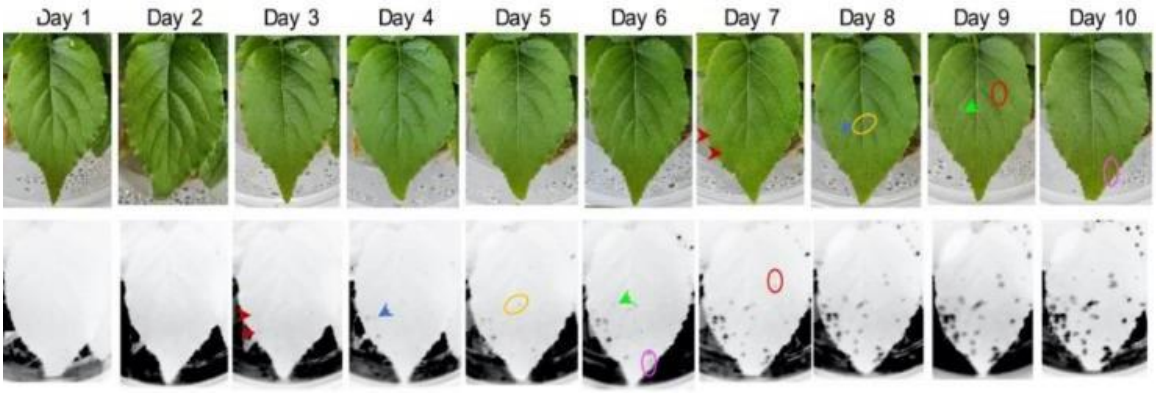
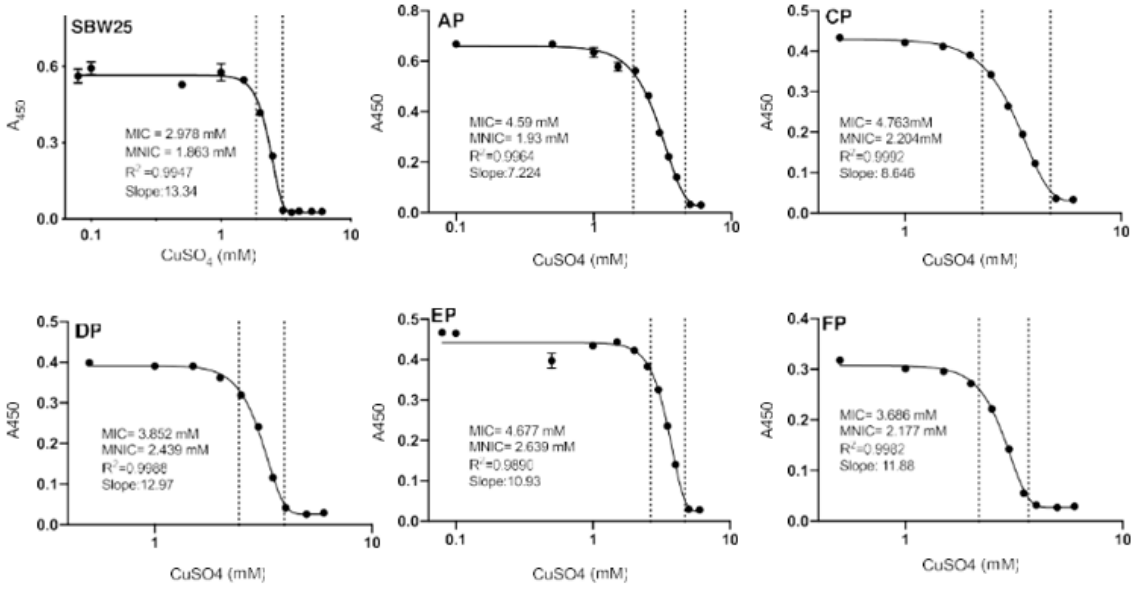
Appendix

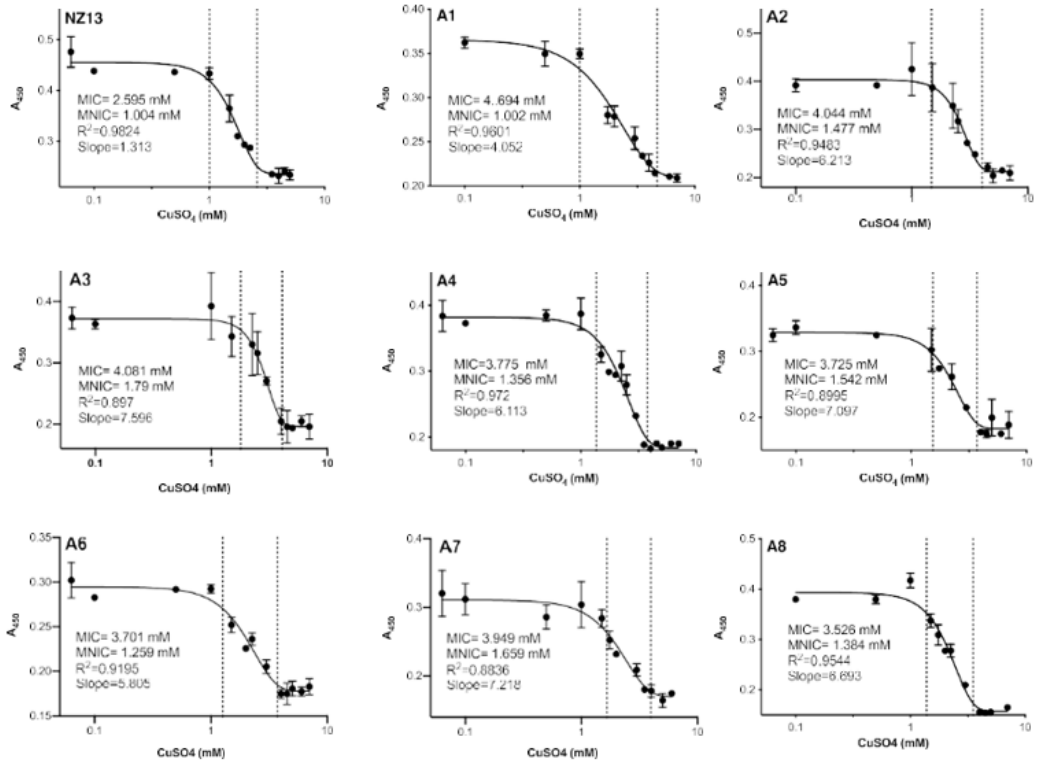
Appendix 1: Investigating the relationship between bioluminescence signals and bacterial spots on young kiwifruit plants. Young Hort16A kiwifruit plants were flood-inoculated with PnptII-lux-labelled NZ13 culture. Bioluminescence signals (captured by CCD camera) and bacterial spots (observed using the naked eye) were compared daily for 10 constitutive days.



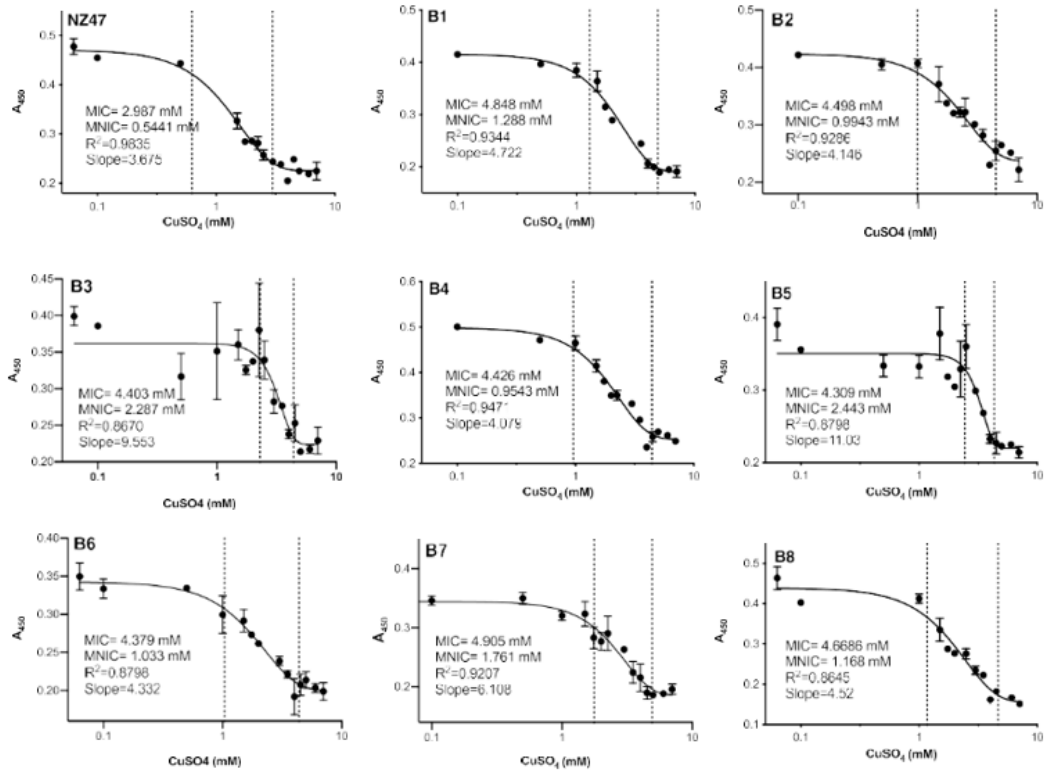
Appendix 2: The $CuSO_4$ MIC and MNIC fitting curves of copper-selected and wildtype *P. fluorescens* SBW25 strains. The A_{450} data from growing assays at the 9-hr. time-point was fitted to the Gompertz-MIC model to calculate the MIC and MNIC of $CuSO_4$.



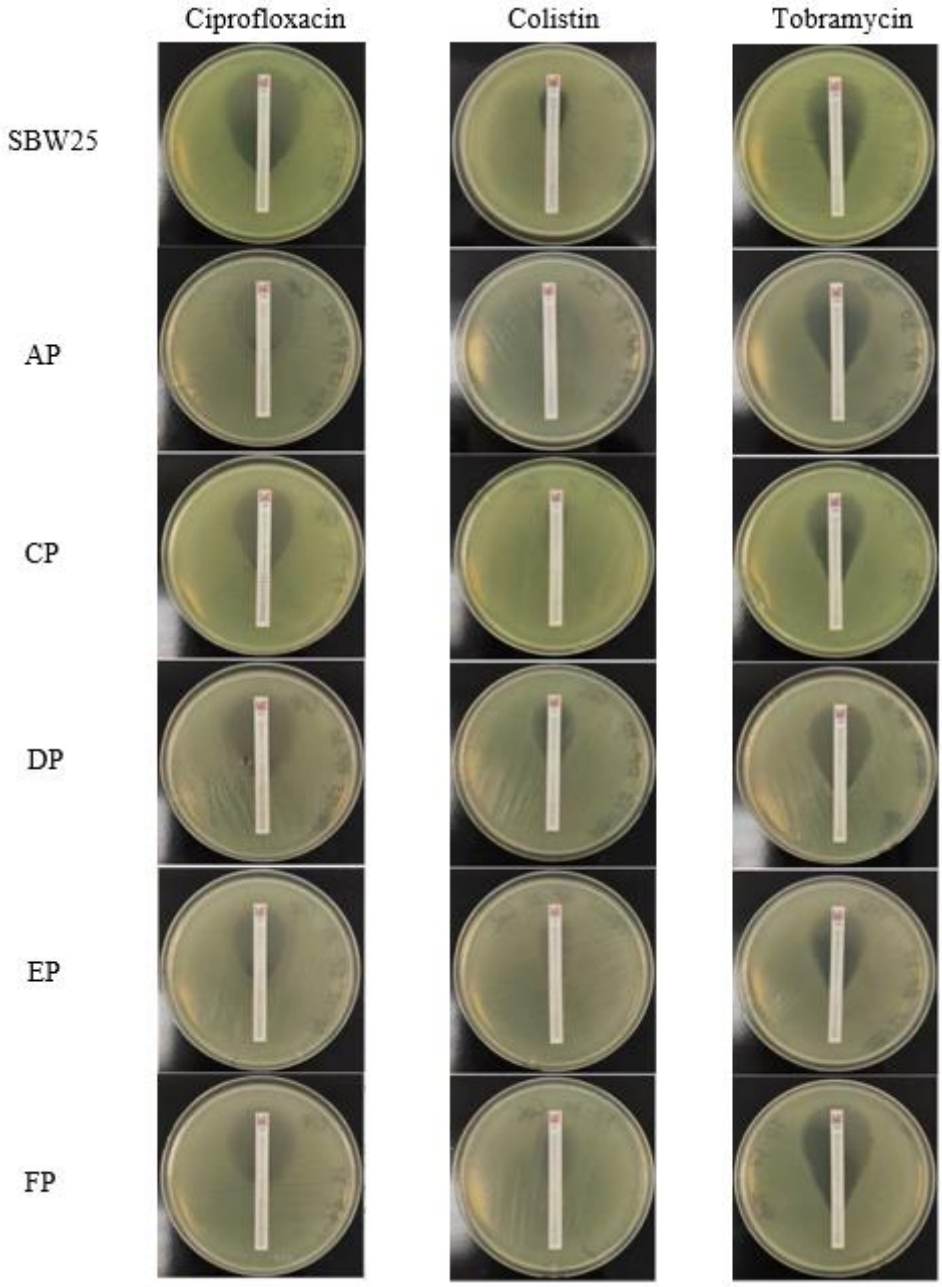
Appendix 3: The $CuSO_4$ MIC and MNIC fitting curves of copper-selected and wildtype *Psa* NZ13 strains. The A_{450} data from growing assays at the 14-hr. time-point was fitted to the Gompertz-MIC model to calculate the MIC and MNIC of $CuSO_4$.



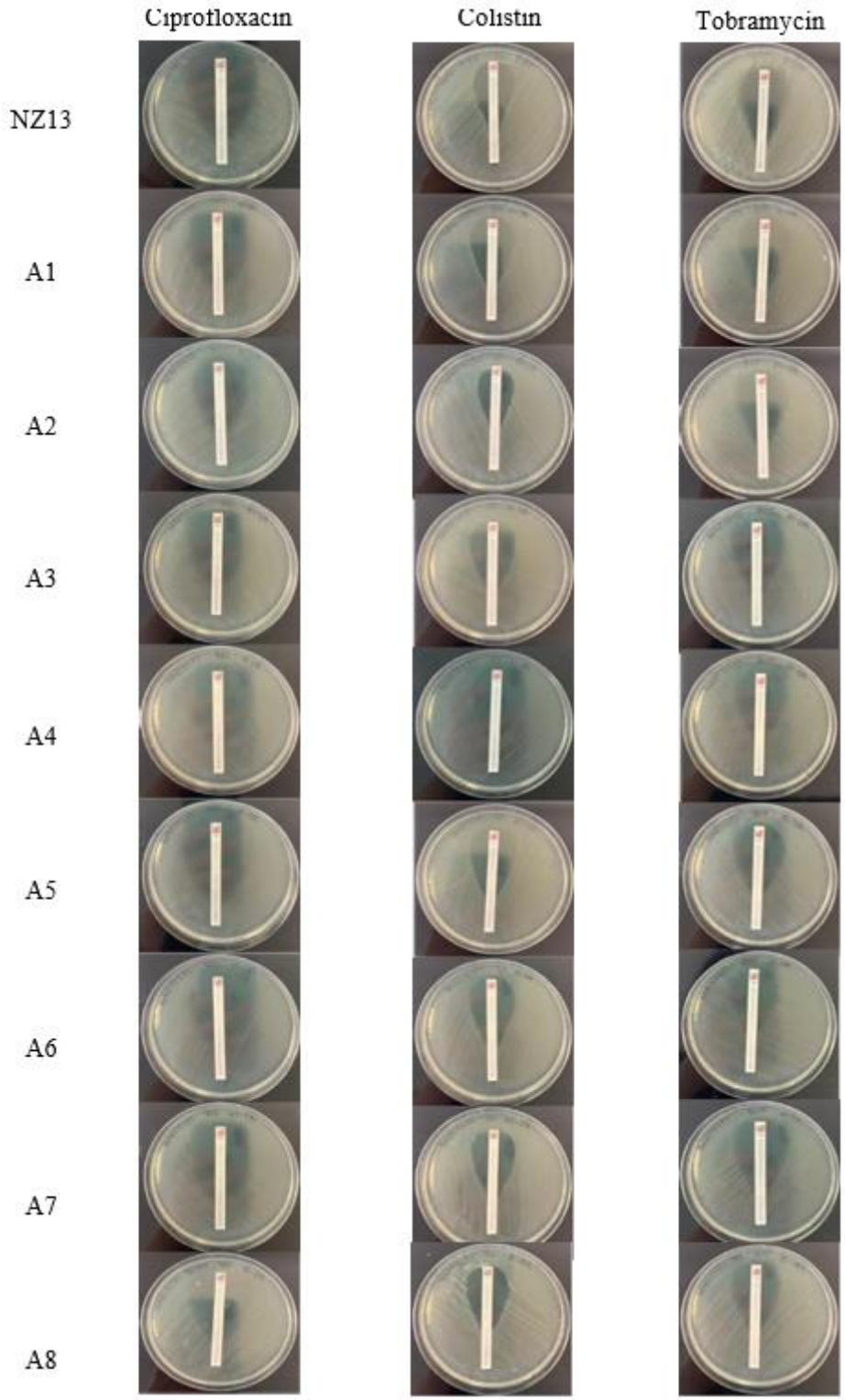
Appendix 4: The $CuSO_4$ MIC and MNIC fitting curves of copper-selected and wildtype *Psa* NZ47 strains. The A_{450} data from growing assays at the 14-hr. time-point was fitted to the Gompertz-MIC model to calculate the MIC and MNIC of $CuSO_4$.

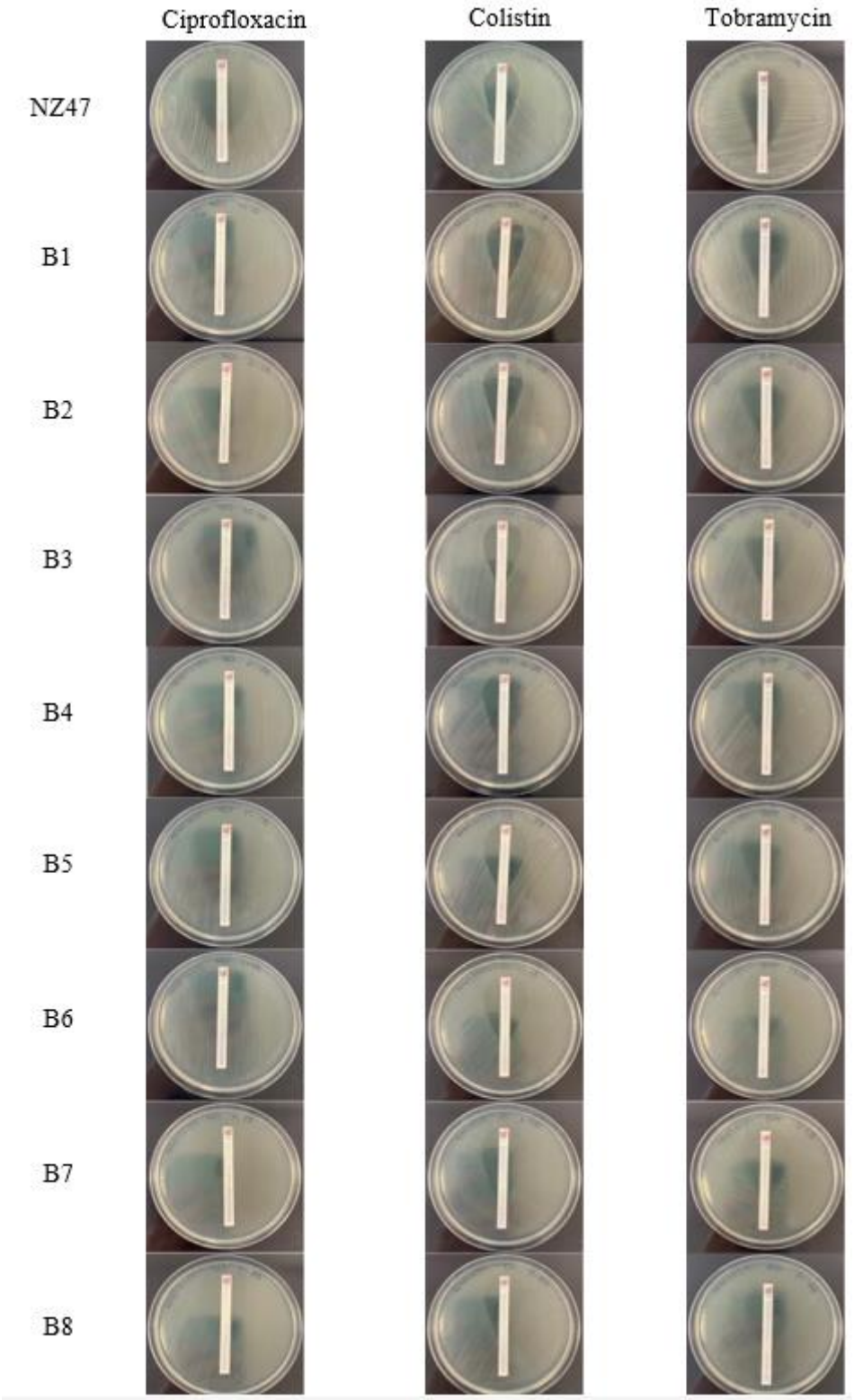


Appendix 5: Photos of antibiotic strip assays for copper-selected *P. fluorescens* SBW25 strains. Ciprofloxacin, colistin and tobramycin resistance were assessed by Liofilchem antibiotic strips. The MICs were examined via the intersection between the strip and the inhibitory zone on inoculated LB agar plates.



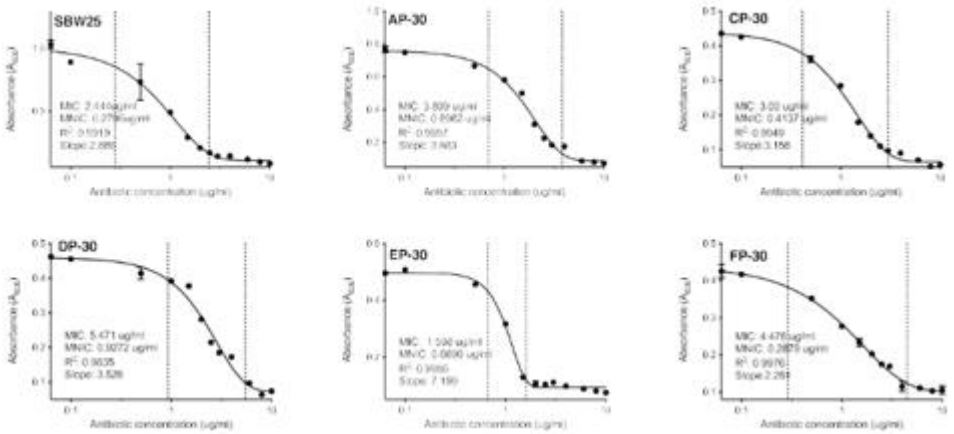
Appendix 6: Photos of antibiotic strip assays for copper-selected *Psa* strains. Ciprofloxacin, colistin and tobramycin resistance were assessed by Liofilchem antibiotic strips. The MICs were examined via the intersection between the strip and the inhibitory zone on inoculated LB agar plate.



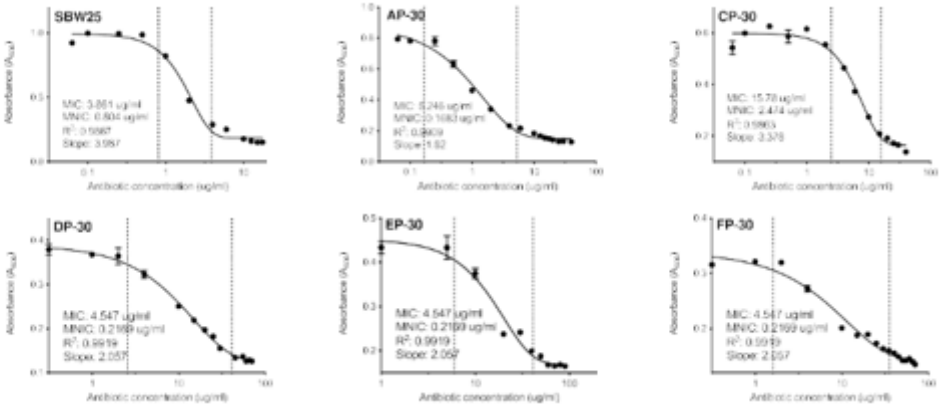

Ciprofloxacin
Colistin
Tobramycin
NZ47
B1
B2
B3
B4
B5
B6
B7
B8

Appendix 7: The antibiotic MIC fitting curves of copper-selected SBW25 and its ancestral strains. Bacterial cultures were grown in LB supplemented with varying concentration of antibiotics. The growth was monitored using a plate-reader and A600 data after 9 hrs. of incubation was used for fitting the Gompertz-model.

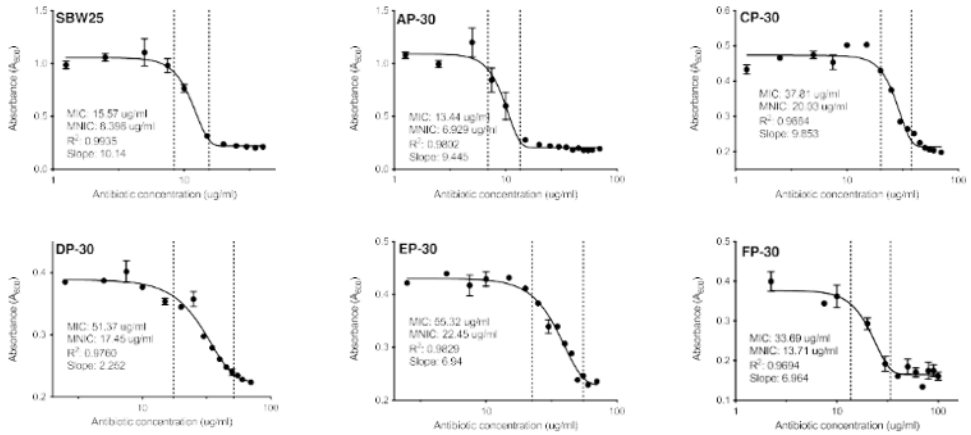
Kanamycin



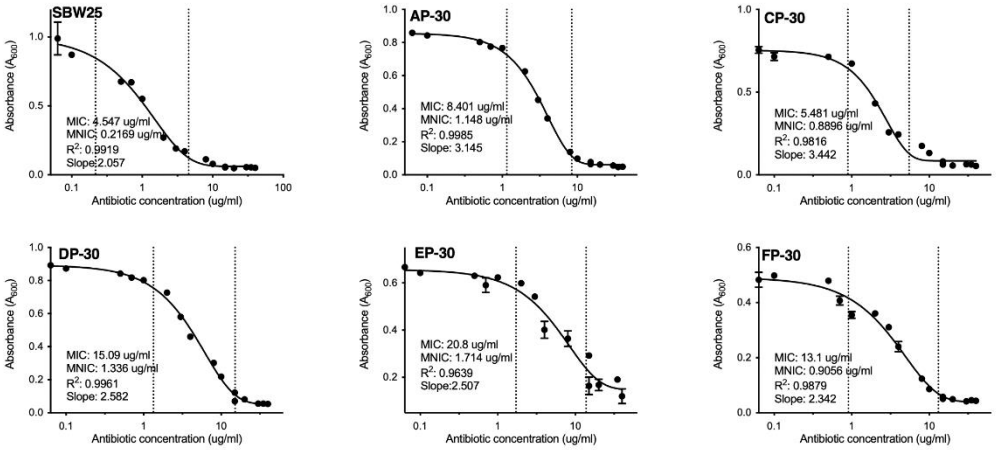
Gentamycin



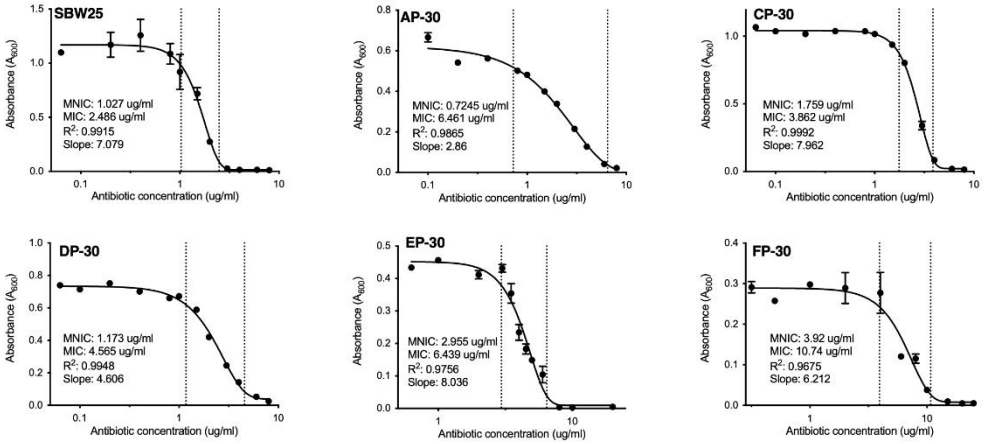
Nalidixic acid



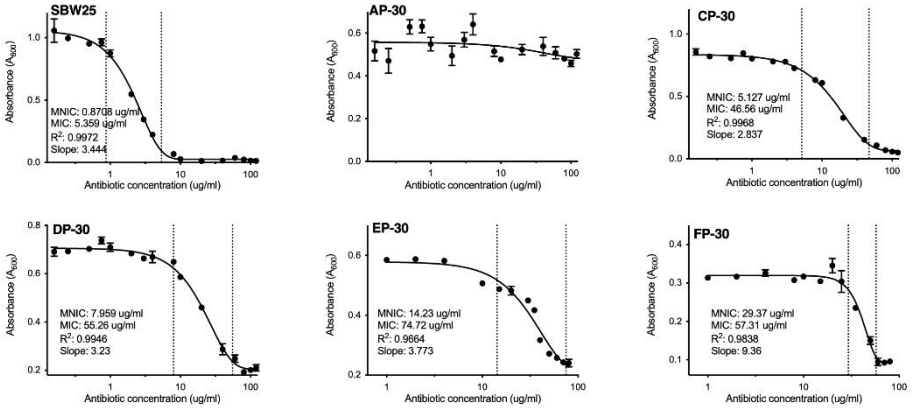
Tobramycin



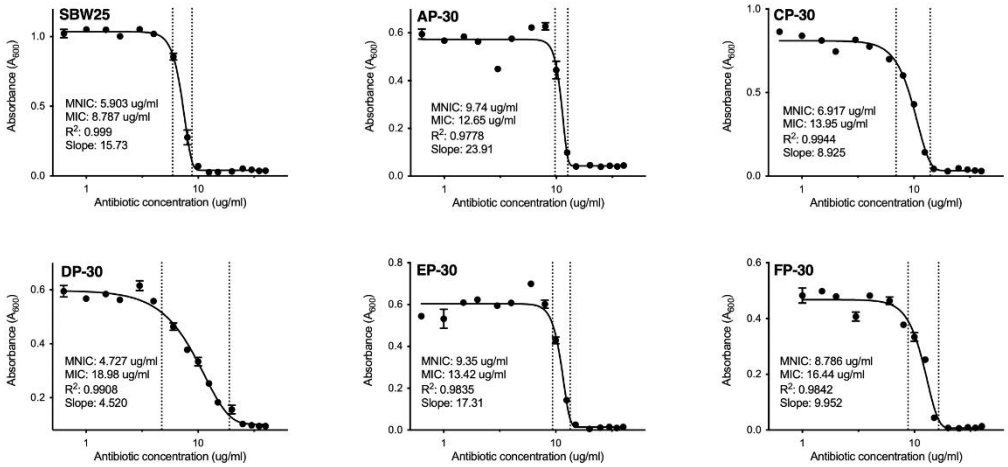
Tetracycline



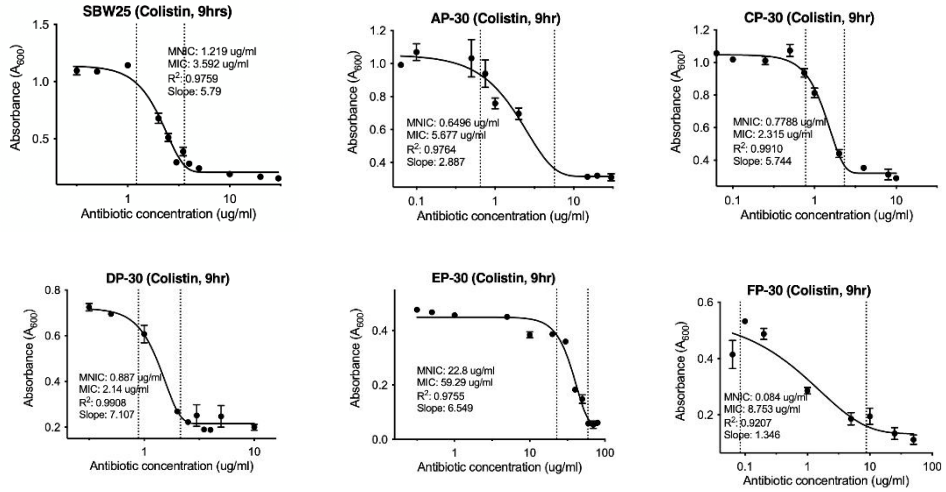
Streptomycin



Rifamycin



Colistin



Appendix 8: Genome sequencing of *P. fluorescens* SBW25-derived strains. Five SBW25-derived strains were subjected to genome re-sequencing and mapped to reference genome NC_012600.1. A set of mutations in knowngene products were identified for each strain and indicated by tick sign in the table.

Product	Locus/ Location	AP	CP	DP	EP	FP
50S ribosomal protein L1	PFLU5537	mismatch	A86T(L29Q)		C20T(R7H)	C76T(A26T)
CopS	PFLU1575	mismatch	mismatch	A830C(L277R)	mismatch	555(C)5->(C)6, Frame shift
(p)ppGpp synthetase-hydrolase, SpoT	PFLU5995	1088C(V363A)				
Promoter region of PFLU3804 (infA)	4,197,116	G -> A				
putative plasmid maintenance system killer protein	PFLU1109	C154T(R52W)				
hypothetical protein	PFLU3999			C330T(A110A)		
putative ferric alcaligin siderophore receptor	PFLU3378			C368T(S123F)		
putative two-component response regulator	PFLU3074			470TCGACA Deletion		
sensor kinase protein	PFLU2723			T1085G(V362G)		
succinate dehydrogenase flavoprotein subunit SdhA	PFLU1818			C359T(P120L)		
AraC family transcriptional regulator	PFLU2563				T612C(E204E)	
LysR family transcriptional regulator	PFLU2442				C166T(E56K)	
heat shock protein HtpX	PFLU1730				731CA Deletion, frame shift	
F0F1 ATP synthase subunit gamma AtpG	PFLU6119					G802A(R268C)
putative racemase	PFLU3789					G632A(A211V)
maltooligosyl trehalose synthase	PFLU3367					G616T(D206Y)
-	1,788,167					G -> A
-	1,788,159					CCGCTAT-> GTTTGTA
hypothetical protein	PFLU0589					C3243T(S1081S)
Genome size (bp)		6,758,453	6,735,975	6,755,354	6,729,753	6,729,496
Coverage means		161.7±25.9	159.6±26.2	163.7±26.9	156.2±25.7	157.4±26.5
Total no. of mutations		5	2	6	5	8

Appendix 9: Genome sequencing of *Psa* NZ13-derived strains. Eight NZ13-derived strains were subjected to genome re-sequencing and mapped to reference genome NZ_CP011972.2. A set of mutations in known gene products were identified for each strain and indicated by tick sign in the table.

Product	Locus/ Location	A1	A2	A3	A4	A5	A6	A7	A8
EnvZ	IYO_RS01860		G440T(S147I)	G1009A (A337T)	G67A(V23M)	T119C(V40A) G1078A(V360M)			
OmpR	IYO_RS01855			C58T(P20S)					G268T (A90S)
protease HtpX	IYO_RS09125	C402A (E134D)						GCGTTG(733) deletion	
HutC	IYO_RS26430			A517C (T173P)					
Promoter region of hutC	5,891,396						G -> A		
SecD	IYO_RS06970	C1811A (T604N)							
3-alpha- hydroxysteroid dehydrogenase	IYO_RS16580		G320A (A107V)						
peptidoglycan- binding LysM familyprotein	IYO_RS07750					C701T (A234V)			
cytochrome ubiquinol oxidase subunit I	IYO_RS06510						A224G (D75G)		
OprD family porin	IYO_RS28050							C209T (G70D)	
penicillin-binding protein 1A	IYO_RS26235								T56G(L19R)
methyl-accepting chemotaxis protein	IYO_RS13675	C1442G (T481S)							
polyphosphate:AMP phosphotransferase	IYO_RS20380		A511G (L171L)						
thioesterase	IYO_RS13845		C593T (G198D)						
peptidase S9	IYO_RS08475		G2047A (Q683Q)						
MFS transporter	IYO_RS04225		C812T (P271L)						
SulP family anion transporter	IYO_RS26860			C405T (V135V)					
PutA,transcriptional regulator	IYO_RS25595			C735T (G245G)					
MpaB oxygenase	IYO_RS23695			G241A (D81N)					
RNA-binding transcriptional accessory protein	IYO_RS01850			A159G (H53H)					
paraquat-inducible protein B	IYO_RS12360				C138T (W46W)				
lauroyl acyltransferase	IYO_RS19980					C420A (A140A)			
non-ribosomal peptide synthetase	IYO_RS13865						C3255T (E1085E)		
TetR family transcriptional regulator	IYO_RS12405						C90G (A30A)		
ribonuclease E	IYO_RS19850							G298A (E100K)	
ribonuclease PH	IYO_RS00760								A61C(Y21D)
-	580,325		G -> T						
-	576,831							A -> G	
-	1,023,430 4,247,547								(CCAATCA A)4 -> (CCAATCA A)5
Genome size (bp)		6,635,565	6,599,180	6,581,330	6,590,620	6,599,832	6,575,611	6,571763	6,566,684
Coverage means.		152.7± 37.1	150.2± 31.8	164.9± 52.7	161.9± 57.5	153.2± 37.1	157.9± 42.8	154.3± 33.8	155.2± 41.4
Total no. of mutations		8	11	11	6	8	8	7	9

Appendix 10: Genome sequencing of *Psa* NZ47-derived strains. Eight NZ47-derived strains were subjected to genome re-sequencing and mapped to reference genome NZ_CP017011.1. A set of mutations in known gene products were identified for each strain showing in the table. Genes highlighted in grey are common to all strains.

Product	Locus/ Location	B1	B2	B3	B4	B5	B6	B7	B8
MscS	PsaNZ47_RS30470				G261A (L87L)				
adenosylhomocysteine	PsaNZ47_RS27835				A731G (D244G)				
ATP-helicase	PsaNZ47_RS22375				T2471G (N824T)				
-	4,232,969				A -> G				
-	4,232,963				TCAG -> CACT				
nucleoside hydrolase	PsaNZ47_RS18785				A806G (V269A)				
ImpK	PsaNZ47_RS14555				T60A (H20Q)				
-	3,411,029				T -> C				
-	1,142,104				C -> G				
EnvZ	PsaNZ47_RS02015		C89T(S30F)	G1109A (G370D)	C89T(S30F)	G1211A (R404H)		C1316G (A439G) C52A(R18S	
HtpX	PsaNZ47_RS09805	C402A (E134D)		C402A (E134D)			429TGCCA GTACG deletion		381CC deletion
KefA	PsaNZ47_RS27535		C345T (Y115Y)		C345T (Y115Y)				
-	743,504					(GTCATT GC)5 -> (GTCATT GC)6		(GTCATT GC)5 -> (GTCATT GC)6	
-	2,585,769	(TGTATCT GAAT)3 -> (TGTATCT GAAT)2							
-	5,061,397		A -> C						
-	4,034,962		T -> C						
-	1,023,430			(CCAATC AA)4 -> (CCAATC AA)5					
DNA-RNA polymerase	PsaNZ47_RS02925			A2626T (S876C)					
16SrRNAmethyltransferase	PsaNZ47_RS28330				A232C (T78P)				
adenylylsulfate kinase	PsaNZ47_RS24150				C1654T (E552K)				
phosphate acyltransferase	PsaNZ47_RS21315				438(C)5 -> (C)6				
Transposase	PsaNZ47_RS04025				A300G (A100A) G315A (F105F) A339G (H113H) A348G (L116L)				
HutC	PsaNZ47_RS28410					C191T (A64V)			
ligand-gated channel	PsaNZ47_RS14920					C1794T (L598L)			
hydroxyglutarate aldolase	PsaNZ47_RS06940					G568T (V190F)			
TerA	PsaNZ47_RS25620						G873C (G291G)		
NADH-oxidoreductase H	PsaNZ47_RS12280						C122A (G41V)		
LysM	PsaNZ47_RS08315						G426A (K142K)		
-	301,883						(C)13 -> (C)14		
hypothetical protein	PsaNZ47_RS23890							C775T (D259N)	
membrane protein	PsaNZ47_RS20040							G57A (D19D)	
LysR	PsaNZ47_RS17110							T717C (D239D)	
xylose isomerase	PsaNZ47_RS15815							A1178C (F393C)	
prevent-host-death protein	PsaNZ47_RS09015							Mismatch	
Urease	PsaNZ47_RS04915							G834A (G278G)	
DedA	PsaNZ47_RS28840								354ACCC deletion
Peptidase	PsaNZ47_RS23760								157(CCGG CC)2->(C CGGCC)3
-	4,069,379								C -> T
Genome size (bp)		6,560,644	6,576,650	6,641,002	6,573237	6,565,792	6,562,182	6,576,896	6,558,961
Coverage mean		161.7± 58.3	155.3± 30.4	148.0± 49.1	150.5± 30.0	157.4± 44.6	160.7± 46.0	163.4± 41.2	159.2± 52.8
Total no. of mutations		11	13	13	18	14	14	17	13